**STIC-ILL**

**From:** Davis, Minh-Tam 1642
**Sent:** Monday, August 29, 2005 2:41 PM
**To:** STIC-ILL
**Subject:** REPRINT REQUEST FOR 10/069973



1) INVOLVEMENT OF CYCLOPHILIN - D **IN THE ACTIVATION OF A MITOCHONDRIAL PORE BY CA2+ AND OXIDANT STRESS** (Abstract Available)
Author(s): TANVEER A; VIRJI S; ANDREEVA L; TOTTY NF; HSUAN JJ; WARD JM; CROMPTON M
Corporate Source: UNIV LONDON UNIV COLL,DEPT BIOCHEM & MOLEC BIOL,GOWER ST/LONDON WC1E 6BT//ENGLAND/; UNIV LONDON UNIV COLL,DEPT BIOCHEM & MOLEC BIOL/LONDON WC1E 6BT//ENGLAND/; LUDWIG INST CANC RES/LONDON W1P 8BT//ENGLAND/
Journal: EUROPEAN JOURNAL OF BIOCHEMISTRY, **1996** , V238, N1 (MAY), P166-172
ISSN: 0014-2956

2) **The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury.**
Halestrap A P
Department of Biochemistry, University of Bristol, U.K.
Biochemical Society symposium (ENGLAND) **1999** , 66 p181-203, ISSN 0067-8694 Journal Code: 7506896
Publishing Model Print
Document type: Journal Article; Review

3) **mport and processing of heart mitochondrial** cyclophilin D .
Johnson N; Khan A; Virji S; Ward J M; Crompton M
Department of Biochemistry and Molecular Biology, University College London, London, UK.
European journal of biochemistry / FEBS (GERMANY) Jul **1999** , 263 (2) p353-9, ISSN 0014-2956 Journal Code: 0107600

4) **The mitochondrial permeability transition pore and its role in cell death.**
Crompton M
Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K. m.crompton@biochemistry.ucl.ac.uk
Biochemical journal (ENGLAND) Jul 15 **1999** , 341 ( Pt 2) p233-49, ISSN 0264-6021 Journal Code: 2984726R
Publishing Model Print

5) **The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins.**
Marzo I; Brenner C; Zamzami N; Susin S A; Beutner G; Brdiczka D; Remy R; Xie Z H; Reed J C; Kroemer G
Centre National de la Recherche Scientifique, Unite Propre de Recherche 420, F-94801 Villejuif, France.
Journal of experimental medicine (UNITED STATES) Apr 20 **1998** , 187 (8) p1261-71, ISSN 0022-1007 Journal Code: 2985109R

6) Cyclophilin - D **binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore.**
Crompton M; Virji S; Ward J M
Department of Biochemistry and Molecular Biology, University College London, UK. m.crompton@bsm.biochemistry.ucl.ac.uk
European journal of biochemistry / FEBS (GERMANY) Dec 1 **1998** , 258 (2) p729-35, ISSN 0014-2956 Journal Code: 0107600
Publishing Model Print

REVIEW ARTICLE

# The mitochondrial permeability transition pore and its role in cell death

Martin CROMPTON[1]

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

This article reviews the involvement of the mitochondrial permeability transition pore in necrotic and apoptotic cell death. The pore is formed from a complex of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase and cyclophilin-D (CyP-D) at contact sites between the mitochondrial outer and inner membranes. *In vitro*, under pseudopathological conditions of oxidative stress, relatively high $Ca^{2+}$ and low ATP, the complex flickers into an open-pore state allowing free diffusion of low-$M_r$ solutes across the inner membrane. These conditions correspond to those that unfold during tissue ischaemia and reperfusion, suggesting that pore opening may be an important factor in the pathogenesis of necrotic cell death following ischaemia/reperfusion. Evidence that the pore does open during ischaemia/reperfusion is discussed. There are also strong indications that the VDAC–adenine nucleotide translocase–CyP-D complex can recruit a number of other proteins, including Bax, and that the complex is utilized in some capacity during apoptosis. The apoptotic pathway is amplified by the release of apoptogenic proteins from the mitochondrial intermembrane space, including cytochrome *c*, apoptosis-inducing factor and some procaspases. Current evidence that the pore complex is involved in outer-membrane rupture and release of these proteins during programmed cell death is reviewed, along with indications that transient pore opening may provoke 'accidental' apoptosis.

Key words: apoptosis, $Ca^{2+}$, cyclophilin, necrosis, oxidative stress.

## INTRODUCTION

When respiring mitochondria take up $Ca^{2+}$ in the presence of $P_i$ and external adenine nucleotides, the accumulated $Ca^{2+}$ is retained indefinitely. But in the absence of adenine nucleotides the accumulated $Ca^{2+}$ is subsequently released along with other matrix solutes. The lesion has been identified as a $Ca^{2+}$-dependent pore in the mitochondrial inner membrane, generally referred to as the permeability transition (PT) pore. Until recently, any biological significance of the PT pore was obscure, and the field of research lay fallow for some time. But recent developments have brought interest in the area to the fore. Among these are its perceived relevance to ischaemia-related disease, the discovery of the active involvement of mitochondria in apoptosis, and the emerging interactions between PT pore components and regulatory proteins of apoptosis. Taken together, these provide a case for PT pore involvement in cell death. This review does not attempt a comprehensive documentation of information relating to the PT pore. Rather, it focusses on those strands of research that address the question of a link between the PT pore and cell death and that have made the field a topical one.

## 1 $Ca^{2+}$ CYCLING AND THE CONTROL OF MITOCHONDRIAL $Ca^{2+}$

PT-induced mitochondrial dysfunction is a consequence of mitochondrial $Ca^{2+}$ overload. It is appropriate, therefore, to define the conditions under which the overload takes place. In the following, mitochondrial $Ca^{2+}$ overload is discussed against a background of how mitochondrial $Ca^{2+}$ is controlled normally, and how it can lead to PT pore opening.

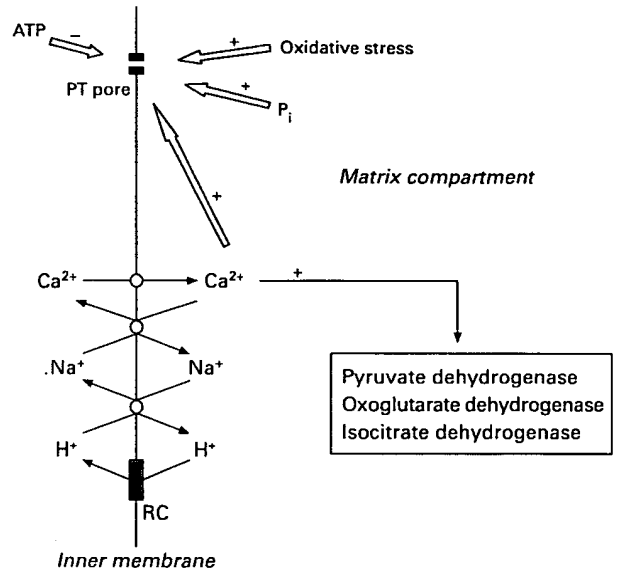
### 1.1 Physiological $Ca^{2+}$ cycling

The control of intramitochondrial $Ca^{2+}$ by $Ca^{2+}$ cycling has been recognized for over 20 years. $Ca^{2+}$ enters mitochondria electrophoretically via the $Ca^{2+}$ uniporter [1] and exits by exchange with $Na^+$ on the $Na^+/Ca^{2+}$ carrier [2,3]. These two transport systems together with the $Na^+/H^+$ antiporter establish a transport cycle that mediates slow, continuous cycling of $Ca^{2+}$ across the inner membrane driven by the respiratory-chain expulsion of $H^+$ ([1,2]; reviewed in [4,5]) (Scheme 1). A second carrier for $Ca^{2+}$ efflux, mediating $Ca^{2+}/H^+$ exchange [6], is sometimes invoked to explain $Ca^{2+}$ cycling in mitochondria with low $Na^+/Ca^{2+}$ carrier activity [7]. But the status of this is unclear. Unlike the fluxes mediated by the uniporter and the $Na^+/Ca^{2+}$ carrier, $Na^+$-independent efflux of $Ca^{2+}$ is resistant to lanthanides [8], which typically bind with high affinity to $Ca^{2+}$- binding sites, and would be expected to block a specific carrier for $Ca^{2+}$ efflux. On the other hand, $Na^+$-independent $Ca^{2+}$ efflux is inhibited when energy transduction is uncoupled, implying that an active system may exist [9].

Continuous $Ca^{2+}$ cycling across the inner membrane means that mitochondrial $Ca^{2+}$ is established by the kinetic properties of the uniporter and efflux system(s), rather than merely by the forces driving the fluxes. To illustrate this, $Ca^{2+}$ influx via the uniporter is driven by the $Ca^{2+}$ electrochemical gradient, and the rate of this process increases with increasing membrane potential, but only up to 110 mV or so (isolated liver mitochondria) [5,9]. Above this value, uniporter activity reaches a plateau. As a result, decreases in inner-membrane potential to 110 mV do not produce losses of mitochondrial $Ca^{2+}$ (as might be expected), at least in isolated mitochondria. Whether a similar relation holds *in vivo* is not known.

Abbreviations used: ANT, adenine nucleotide translocase; Aop 1, antioxidant protein 1; CSA, cyclosporin A; CyP, cyclophilin; DISC, death-inducing signalling complex; DOG, 2-deoxyglucose; PT, permeability transition; TMRME, tetramethylrhodamine methyl ester; VDAC, voltage-dependent anion channel; GST, glutathione S-transferase; $O_2^-$ superoxide anion; NEM, *N*-ethylmaleimide; FADD, Fas-activated protein with death domain; DISC, death-inducing signalling complex; DED, death effector domain; CARD, caspase recruitment domain; AIF, apoptosis-inducing factor.

[1] e-mail m.crompton@biochemistry.ucl.ac.uk




**Scheme 1 Physiological and pathological effects of mitochondrial $Ca^{2+}$**

Mitochondrial $Ca^{2+}$ is controlled by a transport cycle driven by the proton pumps of the respiratory chain (RC). The transport cycle is mediated by the $Ca^{2+}$ uniporter, the $Na^+/Ca^{2+}$ antiporter and $Na^+/H^+$ antiporter of the inner membrane. Under physiological conditions, mitochondrial $Ca^{2+}$ controls key regulatory dehydrogenases in the mitochondrial matrix. Under pathological conditions associated with cellular ATP depletion and oxidative stress, mitochondrial $Ca^{2+}$ triggers opening of the PT pore.

The transport cycle enables changes in cytosolic $[Ca^{2+}]$ to be relayed into changes in $[Ca^{2+}]$ in the mitochondrial matrix. Changes in matrix free $[Ca^{2+}]$ are normally within the limits 0.2–10 $\mu$M, which is the effective range for regulation of $Ca^{2+}$-sensitive enzymes in that compartment [10]. These include the key regulatory enzymes of oxidative metabolism, namely pyruvate dehydrogenase, oxoglutarate dehydrogenase and isocitrate dehydrogenase [10] (Scheme 1). By extending the messenger role of $Ca^{2+}$ to the mitochondria, the $Ca^{2+}$-transport cycle allows mitochondrial oxidative metabolism to respond to $Ca^{2+}$-dependent events in the cytosol. This is most obvious in the case of heart muscle contraction, which is driven very largely by aerobic metabolism. When myocardial contractility increases, the increased height and frequency of the cytosolic $Ca^{2+}$ transients leads to an increase in mitochondrial $[Ca^{2+}]$ and consequent activation of the tricarboxylic acid cycle and oxidative phosphorylation [11]. In this way, aerobic ATP synthesis is effectively co-ordinated with ATP usage. Thus increases in cardiac work (adrenaline, electrical pacing) are associated with increased mitochondrial $Ca^{2+}$ and produce proportional (2–4-fold) increases in oxidative phosphorylation (oxygen uptake) without detectable change in the ATP/ADP ratio [11,12]. But when mitochondrial $Ca^{2+}$ uptake in heart cells is blocked (Ruthenium Red) increased work produces a fall in the ATP/ADP ratio as the cell, now unable to exploit $Ca^{2+}$ as a catabolic signal, reverts to adenine nucleotide control of catabolism [12].

This is the basic function of mitochondrial $Ca^{2+}$ transport and, in order to fulfill this role, it seems most probable that the cycle transports $Ca^{2+}$ slowly. Slowness of $Ca^{2+}$ cycling is evident from many approaches. Simulations based on the kinetic properties of the uniporter and $Na^+/Ca^{2+}$ carrier indicate that a single cytosolic $Ca^{2+}$ transient in rat heart (occurring over a 10-fold concentration range) would produce little change (i.e. < 5%) in mitochondrial $Ca^{2+}$, since the event is simply too short in relation to the kinetic behaviour of the transport cycle [4,5,13]. Rather, a succession of transients over 1 min or so is needed for mitochondrial $Ca^{2+}$ to establish a quasi-steady state with respect to a particular transient height and frequency. This is borne out by the appropriate measurements. Thus, when myocardial contractility (cytosolic $Ca^{2+}$ transient height and frequency) is increased (adrenaline), there is an immediate fall in the ATP/ADP ratio, followed by a recovery over 1 min [13]. This is exactly the behaviour predicted if it takes mitochondrial $Ca^{2+}$ about 1 min to establish a new quasi-steady state; initially the tricarboxylic acid cycle is activated by increased ADP (acting on the pyruvate, oxoglutarate and isocitrate dehydrogenases), but mitochondrial $Ca^{2+}$ gradually takes over as activator as its concentration increases. Slowness was also indicated by the studies of Miyata et al. [14], who loaded rat cardiomyocytes with the $Ca^{2+}$ indicator Indo-1, and then selectively quenched the cytosolic $Ca^{2+}$ signal with $Mn^{2+}$, enabling changes in mitochondrial $Ca^{2+}$ to be revealed. Large-scale beat-to-beat changes in cytosolic $Ca^{2+}$ were relayed to the mitochondrial matrix as a very largely damped-out ripple of mitochondrial $Ca^{2+}$. Again, about 1 min was required for mitochondrial $Ca^{2+}$ to establish a new quasi-steady state when the height and frequency of the cytosolic $Ca^{2+}$ transients was changed. Slowness was confirmed by another approach in which cytosolic Indo-1 was selectively expelled via probenicid-sensitive anion pumps in the cardiomyocyte plasma membrane, allowing residual mitochondrial Indo-1 to be used for the measurement of mitochondrial $Ca^{2+}$ [15]. Again the measured beat-to-beat changes in mitochondrial $Ca^{2+}$ were negligible. Slow $Ca^{2+}$ cycling yields several consequences. First, it imposes little energy drain on the cell; this is an essential condition, since the uniporter is not regulated by any acute on/off mechanism, and $Ca^{2+}$ cycling occurs all the time. Secondly, the slow response enables the large oscillations in cytosolic $[Ca^{2+}]$ to be translated into relatively constant levels of mitochondrial $Ca^{2+}$ that reflect the mean cytosolic $[Ca^{2+}]$ over a short period of time. This allows a relatively steady response of mitochondrial oxidative metabolism to energy demands imposed by $Ca^{2+}$-dependent events in the cytosol. Thirdly, the slow response of the transport cycle ensures that it does not interfere (too much) with the cytosolic $Ca^{2+}$ transients on which the physiological performance of the tissue may depend.

In non-excitable tissues, the cytosolic $Ca^{2+}$ transients caused by hormones are of longer duration and lower frequency. In liver, for example, each transient induced by adrenaline, vasopressin or angiotensin lasts for 10–20 s when measured as a global, cellular event. In reality, each transient is propagated as a wave across the cell, so that local changes in cytosolic $[Ca^{2+}]$ occur within a shorter time span. Nevertheless, the longevity of each transient means that mitochondrial $Ca^{2+}$ would be expected to oscillate much more than in, for example, heart. Thus, in one study on hepatocytes, the large oscillations in cytosolic $[Ca^{2+}]$ (e.g. 400% over basal) produced 100% changes in mitochondrial $[Ca^{2+}]$ [16]. However, measurements of mitochondrial $Ca^{2+}$ reveal a broad spectrum of agonist-induced changes according to cell type and method of measurement (reviewed in [17]). In part this may reflect heterogeneous responses of mitochondria within any single cell, depending on their proximity to the $Ca^{2+}$ release channels of endoplasmic reticulum. Juxtaposed mitochondria experience high localized $[Ca^{2+}]$ and show correspondingly larger changes in matrix free $[Ca^{2+}]$ (see section 4.3). This heterogeneity among mitochondria may mean that, whereas some mitochondria are primarily engaged in regulating oxidative metabolism in accordance with energy demands, and respond in the 'heart

mode' (see above), others exercise a different $Ca^{2+}$-dependent role.

### 1.2 $Ca^{2+}$ cycling in cell injury

It is sometimes argued that pathological situations involving cellular $Ca^{2+}$ overload are associated with sufficiently rapid mitochondrial $Ca^{2+}$ cycling to produce an intolerable energy drain and that this contributes to the pathogenesis of the injury [18,19]. It is possible to estimate the extent of energy dissipation, at least on a global cellular basis. The rate of $Ca^{2+}$ cycling cannot be greater than the activity of the $Ca^{2+}$ uniporter. In rat heart, for example, uniporter activity will vary according to cytosolic free [$Ca^{2+}$] during the contraction–relaxation cycle, but with a mean cytosolic free [$Ca^{2+}$] of 1 $\mu$M (for example) the mean rate of $Ca^{2+}$ cycling has been estimated to be < 1nmol of $Ca^{2+}$/min per mg of mitochondrial protein [4]. If heart contains < 100 mg of mitochondrial protein/g wet wt. (from the cytochrome *c* contents of rat heart and rat heart mitochondria [20,21]), and with 9 $H^+$ extruded (or 4.5 $Ca^{2+}$ ions taken up) for each O atom reduced, then $Ca^{2+}$ cycling under physiological conditions would account for < 15 nmol of oxygen/min per g wet wt., which is less than 0.2% of the tissue oxygen consumption (10 $\mu$mol of oxygen/min per g wet wt. under 80 cm of water [22]). Put another way, the exchange of $Ca^{2+}$ between mitochondria and cytosol each second (< 2 nmol of $Ca^{2+}$/g wet wt.) is less than 1% of the $Ca^{2+}$ removed from the cytosol during relaxation (around 200 nmol of $Ca^{2+}$/g wet wt.) [4]. Thus even a 10-fold increase in mean cytosolic $Ca^{2+}$ under pathological conditions would be expected to increase respiration only by 2% or so. Bearing in mind that heart can increase its respiration at least 4-fold (electrical pacing; section 1.2), it is clear that energy dissipation by mitochondrial $Ca^{2+}$ cycling is negligible when calculated on a global cellular basis. Equally, if pathological insults (e.g. oxidant stress [18,19]) promote mitochondrial $Ca^{2+}$ efflux, $Ca^{2+}$ cycling would only increase inasmuch as cytosolic [$Ca^{2+}$] and uniporter activity increased. Any such increase is likely to be small and temporary, since mitochondria normally have about the same $Ca^{2+}$ content as the cytosol [10,13]. To conclude, although mitochondrial $Ca^{2+}$ cycling dissipates energy, it seems unlikely to be a significant factor determining cell viability.

### 1.3 Mitochondrial $Ca^{2+}$ overload and the PT pore

In contrast with the physiological behaviour of the mitochondrial $Ca^{2+}$ transport cycle (section 1.1), a quite different response of the cycle emerges when the changes in cytosolic [$Ca^{2+}$] are very slow or maintained. Ischaemia and oxidative stress, for example, bring about a slow, progressive increase in resting (basal) cytosolic free [$Ca^{2+}$]. In this case, the cycle has the time to establish a true steady-state distribution of $Ca^{2+}$ across the inner membrane (defined as equal rates of $Ca^{2+}$ influx and efflux). Each increment (maintained) in resting cytosolic [$Ca^{2+}$] gives rise to a proportionally greater increase in mitochondrial [$Ca^{2+}$] until, at about 1–3 $\mu$M cytosolic $Ca^{2+}$, mitochondrial $Ca^{2+}$ overload occurs (the mitochondrial $Ca^{2+}$ content tends towards infinite) ([23]; reviewed in [4,6]). This limiting value of extramitochondrial [$Ca^{2+}$] (maintained) consistent with mitochondrial viability has been termed the 'set point' [23] and relates to a pathological rather than physiological state (since a true steady state is not attainable with relatively rapid $Ca^{2+}$ transients). The concept of the set point was derived from work with isolated mitochondria [13,23] and simulations of $Ca^{2+}$ cycle behaviour [4], but mitochondria *in situ* appear to behave in the same way. Miyata et al. [24] used Indo-1-loaded cardiomyocytes (with and without quenching of the cytosolic signal) to estimate mitochondrial (mit) and cytosolic (cyt) [$Ca^{2+}$] during anoxia. The values obtained correspond well with those calculated from influx–efflux kinetics [4], that is: $Ca^{2+}(mit) < Ca^{2+}(cyt) < 250$ nM and $Ca^{2+}(mit) > Ca^{2+}(cyt) > 250$ nM.

Mitochondrial $Ca^{2+}$ overload is a major feature of cell injury. But overload *per se* is probably inocuous. Thus isolated mitochondria can accumulate $Ca^{2+}$ with impunity as long as exogenous adenine nucleotides are supplied and intramitochondrial pyridine nucleotides are maintained in a sufficiently reduced state [6,25]. This applies equally *in vivo*. When the plasma-membrane $Na^+/K^+$ pump in heart cells is inhibited (ouabain), the rise in intracellular $Na^+$ leads to increased cytosolic $Ca^{2+}$, which, in turn, produces > 100-fold increase in mitochondrial $Ca^{2+}$ (electron probe X-ray microanalysis) [26]. Under these conditions, ATP is largely (75%) maintained [27] and the cells remain viable [26]. Similar data have been reported for vascular smooth-muscle cells [28].

In contrast, in the absence of exogenous adenine nucleotides and in the presence of high $P_i$ or peroxides mitochondrial $Ca^{2+}$ overload invariably leads to PT pore opening (Scheme 1; section 3). PT pore opening was first observed by Haworth and Hunter in the late 1970s. The osmotic behaviour of $Ca^{2+}$-plus-$P_i$-treated mitochondria suspended in poly(ethylene glycol)s showed a sharp cut-off in permeability at $M_r$ 1500, consistent with the induction of a large pore of discrete size [29]. Subsequently a rapid-pulsed-flow technique was used to measure [$^{14}$C]solute fluxes directly. There was an inverse relation between permeability and molecular size which, when analysed according to the Renkin equation, indicated a pore radius of 1.0–1.3 nm [30,31]. Since the hydrodynamic radius of poly(ethylene glycol) 1500 is about 1.2 nm [32], the two sets of measurement are in close agreement. Thus the open pore is large enough to admit most metabolites as well as hydrated inorganic ions, including $Ca^{2+}$. There is some evidence for a lower conductance state of the pore. When the PT pore in intact mitochondria is triggered with $Ca^{2+}$ and $P_i$, the inner-membrane potential is initially rapidly collapsed (as $Ca^{2+}$ enters electrophoretically via the uniporter), restored (when $Ca^{2+}$ uptake is complete) and then collapsed again as the PT pore opens [33]. But the second collapse in potential precedes permeability to sucrose, suggesting that the PT pore may open in stages via a lower conductance state (i.e., $H^+$-permeable/sucrose-impermeable).

For many years it was maintained that PT pore opening serves to regulate mitochondrial $Ca^{2+}$ by allowing $Ca^{2+}$ efflux (reviewed in [34]), and the idea recurs to this day [35]. But pore regulation of mitochondrial $Ca^{2+}$ is the antithesis of control by $Ca^{2+}$ cycling. The mitochondrial $Ca^{2+}$ cycle employs $Ca^{2+}$-selective transport systems together with a high protonmotive force to drive the transport cycle (Scheme 1), consistent with cell viability. By contrast, pore opening destroys the protonmotive force. If the basic function of mitochondrial $Ca^{2+}$ is to control the tricarboxylic acid cycle and mitochondrial ATP production (section 1.1), mitochondrial $Ca^{2+}$ itself is unlikely to be regulated by a mechanism (PT pore) that allows tricarboxylic-acid-cycle intermediates to be lost to the cytosol and ATP to be hydrolysed. On these and other grounds we have argued [30,31] that PT pore opening is a critical event leading to $Ca^{2+}$-induced cell death, rather than an ongoing $Ca^{2+}$ control mechanism of viable cells.

The question arises whether the PT pore opens briefly as a physiological means of ridding mitochondria of excess metabolites or ions, in particular $Ca^{2+}$. In fact, transient opening appears to be its normal mode of behaviour. This becomes evident as follows: When pore-activated mitochondria are treated with EGTA to chelate $Ca^{2+}$, pores are closed immediately (<

50 ms [31]), entrapping EGTA in the matrix. If this is done with $Ca^{2+}$/*N*-hydroxyethylethylenediaminetriacetic acid ('HEDTA') buffers and uniporter inhibitors, then the buffer is entrapped; but, with finite free $Ca^{2+}$ in the mitochondrial matrix, pores can reopen, admitting sucrose. In this way it can be shown that pores open transiently at a frequency determined by matrix free $[Ca^{2+}]$ [33,36]. At low flicker frequencies, when only a small fraction of mitochondria have open pores at any point in time, the apparent inner-membrane potential in the whole population is maintained; nevertheless, sucrose eventually enters the entire population, consistent with pore flicker. PT pore flicker has recently been elegantly demonstrated in single mitochondria [37]. Mitochondria immobilized on coverslips were imaged using tetramethylrhodamine methyl ester (TMRME) as membrane-potential indicator; pore opening was triggered by the generation of intramitochondrial reactive oxygen species on photodecomposition of the indicator. Pore flicker was apparent as a transient depolarization inhibited by cyclosporin A (CSA) (section 2.2) and GSH (section 3.4). Depolarizations lasting from a few seconds to over one minute were reversed equally well, indicating that the PT pore opens for varying periods. Single PT pore flicker is also seen in electrophysiological measurements of inner-membrane conductance (section 2.6). In principle, transient pore opening might occur in order to release $Ca^{2+}$ following matrix $Ca^{2+}$ overload [38]. But if so, the mechanism evidently does not work very well, since ouabain-poisoned cells accumulate mitochondrial $Ca^{2+}$ to >100-fold normal [26,28]. There is therefore scant evidence for PT pore opening as a protective mechanism against mitochondrial $Ca^{2+}$ overload.

## 2 PT PORE COMPONENTS

### 2.1 Adenine nucleotide translocase (ANT)

It has long been recognized that PT pore opening is highly susceptible to ligands of the ANT [39]. Of a range of nucleotides, only ANT substrates (ADP, dADP,ATP) were found to interact with the PT pore [40]. ANT operates as a gated pore. When occupied by transportable substrate, it alternates between two conformations in which the ADP/ATP-binding site is either on the matrix side of the inner membrane (m-state) or on the cytoplasmic side (c-state). ANT ligands that bind to the m-state (bongkrekate) inhibit the PT pore, whereas c-state ligands (atractylate, pyridoxal phosphate) activate. This suggests that the c-state conformation is required for PT pore opening.

Whether ANT itself provides the pore structure in the inner membrane has been investigated in reconstituted systems. When purified ANT is incorporated into liposomes, it changes from a selective antiporter to a non-selective pore under high $[Ca^{2+}]$ [41] ($K_{0.5}$ = 100 $\mu$M $Ca^{2+}$ [42]). As with the PT pore $Ca^{2+}$ acts reversibly, although the time required for loss of pore activity of purified ANT on $Ca^{2+}$ removal (20 min [41]) greatly exceeds the time needed for PT pore closure on $Ca^{2+}$ chelation (< 50 ms [31]). Other features of $Ca^{2+}$-treated ANT resemble those of the PT pore. In planar lipid membranes the conductance of the ANT-derived pore was inhibited at low pH, with half-maximal activity at pH 6.2 [41], which is similar to the PT pore [33]. The current–voltage relationship showed a pronounced reversal of conductance at 150–180 mV of both signs, reminiscent of the dependency of the PT pore on inner-membrane potential [41] (section 3.5). The conductance in KCl was not inhibited by ADP [41], in agreement with its lack of effect on pore-mediated $H^+$ and $K^+$ fluxes in mitochondria [43] (but see [44]).

Taken as a whole, these data suggest that the c-state conformation of ANT may be deformed into a non-selective pore by high $[Ca^{2+}]$, in line with original proposals [39]. But any such deformation would need to occur in a highly reversible manner. This becomes clear from pulsed-flow analyses of EGTA-induced pore closure [31]. In $Ca^{2+}$-replete mitochondria pores open and close continuously [33]. On addition of EGTA, mitochondria became $Ca^{2+}$ depleted and pore flicker stops. But this occurs in a heterogeneous fashion, since, at the instant of EGTA addition, some mitochondria will have open pores, others not. Those with open pores are $Ca^{2+}$-depleted and closed permanently. Those with closed pores at the instant of EGTA addition remain $Ca^{2+}$-replete until subsequent pore opening (flicker) allows $Ca^{2+}$ depletion and permanent pore closure. Somewhat paradoxically, therefore, the rate at which PT pores become permanently closed by EGTA depends on the frequency of pore opening. A detailed analysis of this behaviour by rapid flow techniques revealed that ADP markedly increased (i.e. 10-fold) the incidence of pore flicker [31]. Why is this significant? When functioning normally as an antiporter, ANT is only able to change conformation between the m- and c-states in the presence of transportable substrate. This ensures strict antiport. In essence the complimentarity between the transported solute and the intermediate (between m- and c-) states of the carrier provides the binding energy for the conformational change to occur. For example, purified ANT in CHAPS detergent adopts the m-state and is unable to bind atractylate unless ADP is added to catalyse the m-state-to-c-state conversion [45]. Thus ADP catalysis of PT pore flicker means that ANT must be in its native state between flickers; if ANT were in a deformed state (but closed) between flickers, then the binding energy between ADP and ANT would not be available for the conformational change to the c-state to take place, and an open PT pore would not be produced. Pore flicker presumably allows loss of matrix $Ca^{2+}$ and rapid reversion of ANT to its native state.

### 2.2 Cyclophilin-D (CyP-D)

It is quite clear that ANT, by itself, does not provide the PT pore. Thus treatment of solute-loaded submitochondrial particles with high $[Ca^{2+}]$, with or without $Ca^{2+}$ ionophore to allow $Ca^{2+}$ access to both faces of the inner membrane, does not lead to solute release [46,47]. As with ANT, the participation of a further component was first suggested from the effect of the respective ligand. PT pore opening is blocked by CSA at a concentration (approx. 50 pmol/mg of mitochondrial protein) much less than that of ANT (> 1 nmol/mg in heart mitochondria [48]) [49]. Cyclophilin (CyP) involvement was suggested from the similar amounts of bound CSA needed to block the pore and to inhibit the enzymic activity of mitochondrial CyP (below) [47,50], and from the similar relative sensitivities of the PT pore and mitochondrial CyP to CSA analogues [51,52]. In a further approach, a photoactive, radiolabelled CSA derivative was used to tag the CSA 'receptor' [53,54]. Two pore ligands, $Ca^{2+}$ and ADP, were used in conjunction with the derivative to pinpoint the relevant component. These ligands were chosen because they were known to influence CSA interactions with the pore. Thus intramitochondrial $Ca^{2+}$ not only activates the PT pore, but also depresses CSA binding to its 'receptor' on the pore [47,55]. Conversely, ADP promotes CSA binding [56]. When photolabelling was carried out in the presence and absence of these ligands, a number of mitochondrial components became covalently labelled by the CSA derivative, but only photolabelling of CyP-D was promoted by ADP and abolished by $Ca^{2+}$ [53,54], thereby identifying CyP-D as the pore-associated CSA-binding component.

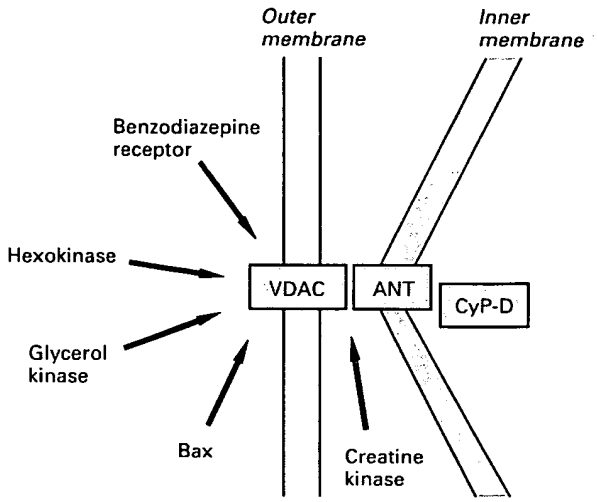
### 2.3 The voltage-dependent anion channel (VDAC)

CyP-D is a water-soluble protein. However, significant quantities of photolabelled CyP-D can be recovered in the membrane fraction [53,54], suggesting that it can bind tightly to an inner-membrane component. The same conclusion may be drawn from the capacity of CSA to block the PT pore in excised patches of the mitochondrial inner membrane [57]; here the CSA-binding component evidently remains bound to the membrane patches. The membrane component was identified using a glutathione S-transferase (GST)–CyP-D fusion protein as affinity matrix. With CHAPS extracts of heart mitochondrial membranes, the affinity-matrix-bound ANT together with about equal amounts of the VDAC [58]. No other CyP-D-binding proteins were detected in the membrane or water-soluble fraction. Since the amount of ANT in heart mitochondria is about 10-fold that of VDAC, the recovery of 1:1 VDAC–ANT complexes probably means that CyP-D selectively targetted the complex rather than free ANT. In a parallel study, using Triton X-100-solubilized membranes of liver mitochondria, ANT alone was bound by the GST–CyP-D fusion protein [59]. The different detergents used in the two studies might explain why VDAC–ANT or ANT alone was recovered. Both CHAPS and Triton X-100 efficiently extract ANT and lead to active reconstituted proteins (as translocase) [60]. But the conformational state of ANT can reflect the detergent used to solubilize it. ANT which has been extracted and purified with CHAPS adopts the m-state, since it binds bongkrekate, but not atractylate [61]. The conformation in Triton X-100 is not known, but some other detergents do yield a protein in the c-state, as judged by ready interaction with atractylate [61]. In any event, from the two studies it seems clear that CyP-D binds to ANT in the VDAC–ANT complex. When the purified VDAC–ANT–CyP-D fusion-protein complex was incorporated into liposomes, addition of $Ca^{2+}$ and $P_i$ brought about PT pore activity (permeability to fluorescein sulphonate), which was blocked by CSA [58]. Both $Ca^{2+}$ and $P_i$ were necessary, as in intact mitochondria [30,33]. Thus the VDAC–ANT–CyP-D complex may well provide the core components of the PT pore.

### 2.4 PT pore topography

ANT is located in the mitochondrial inner membrane and VDAC in the outer. What about CyP-D? CyP-D can be isolated from the matrix fraction of mitochondria [53,62] and is generally assumed to reside in that compartment. But CyP-D purification also yields an N-terminally truncated (eight-residue) form [62]. When CyP are purified from mitochondrial subfractions the full-length (mature) CyP-D is recovered entirely in the inner membrane/matrix fraction and can be assigned to that compartment [54]. The truncated form is recovered from the intermembrane-space fraction, but it is not known whether this represents a genuine species or whether it is a proteolytic artefact produced during purification [54]. However, when *in vitro*-translated $^{35}S$-labelled CyP-D preprotein (i.e. with the N-terminal mitochondrial targetting sequence) is imported into heart mitochondria, it is processed to a single product, which is identifiable as full-length CyP-D [63], suggesting that the truncated form may be 'artefactual'. In any case, only full-length CyP-D is photolabelled by photoactive CSA in an ADP- and $Ca^{2+}$-sensitive manner (above), one of the criteria used to establish CyP-D involvement in the PT pore. Thus the PT pore component appears to be the CyP-D resident in the matrix compartment.

The likely PT pore structure incorporates the complex formed by apposition of VDAC and ANT at contact sites between the mitochondrial outer and inner membranes together with matrix CyP-D (Scheme 2). The VDAC–ANT complex is known to attract other proteins, in particular kinases (e.g. hexokinase, glycerol kinase). The association between these kinases and VDAC–ANT is believed to provide a conduit whereby ATP generated by oxidative phosphorylation is channelled directly to the kinases [64]. ATP utilized by mitochondrially bound hexokinase is derived mainly from oxidative phosphorylation rather than from the cytosol [65]. The properties of the kinase-enlarged complex add further support to the basic VDAC–ANT–CyP-D model. Thus Brdiczka and co-workers have reconstituted pore activity in planar bilayers and in liposomes from preparations that contain VDAC, ANT and CyP-D along with a number of other proteins [42,66–68]. PT pore activity was identified from its sensitivity to $Ca^{2+}$, CSA and atractylate. Preparations containing hexokinase yielded PT pores that were inhibited by the hexokinase substrates glucose and ATP. Hexokinase associates with VDAC at the outer surface of the outer membrane [64], and the fact that glucose was inhibitory is a good indication that the functional pore contained the VDAC–ANT–CyP-D complex rather than ANT–CyP-D alone. In mitochondria, the reaction product glucose 6-phosphate causes desorption of hexokinase from the mitochondrial surface [67]. In the reconstituted PT pore system, glucose 6-phosphate relieved glucose/ATP inhibition, consistent with hexokinase dissociation from the complex [67]. These findings raise the possibility that the locus of inhibition of the PT pore by ATP may be attached hexokinase, rather than ANT (section 3.2). Mitochondrial creatine kinase is located in the intermembrane space and also interacts with VDAC–ANT complexes (Scheme 2). Reconstitutions from preparations containing creatine kinase did not show pore activity unless the creatine kinase octamer was dissociated into dimers. The octameric creatine kinase may suppress the structural transitions necessary for pore formation [66], possibly by impeding VDAC–ANT interactions.




**Scheme 2 PT pore topology**

The basic unit of the PT pore is the VDAC–ANT–CyP-D complex located at contact sites between the mitochondrial inner and outer membranes. Other proteins associate with the complex as indicated.

### 2.5 On the role of CyP-D

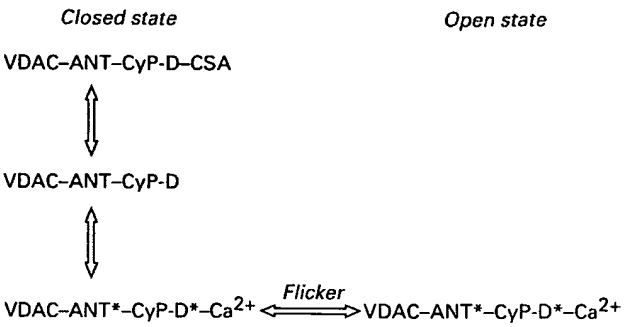
CyP-D is the mitochondrial isoform of a family of CyP proteins that catalyse *cis–trans* isomerization of accessible Xaa–Pro

peptide bonds in proteins. The peptidylprolyl *cis–trans*-isomerase activity is blocked by CSA. The cellular functions of CyP are not well understood. Their catalytic activities point to a role in protein folding and/or conformational change. *In vitro*, CyPs accelerate the refolding of some denatured proteins [69]. *In vivo* it seems that they catalyse the *de novo* folding of some proteins imported into organelles. For example, the folding of newly imported mitochondrial proteins occurs more slowly in yeast mutants lacking mitochondrial CyP [70]. Protein folding in endoplasmic reticulum is inhibited by CSA [71]. In addition it appears that CyPs are recruited into other roles by forming complexes with fully folded, functional proteins. Recognized complexes include the heat-shock protein hsp90 and antioxidant protein, Aop1 (with CyP-A, the cytosolic isoform [72]), the oestrogen receptor (with CyP-40 [73]), the nuclear pore complex (with Nup 358, a CyP domain protein [74]) and the $Ca^{2+}$-modulating Cyp ligand–$Ca^{2+}$-ATPase–calreticulin complex of endoplasmic reticulum (with CyP-B [75]). CyP-D binding to VDAC–ANT provides yet another example of a CyP-containing complex. But, as with the other complexes, the significance of CyP participation in the PT pore complex remains obscure.

If, as does its counterpart in yeast (above), CyP-D catalyses folding of proteins newly admitted into the matrix space, then one assumes that it would exist largely in the free state, free to diffuse to different protein targets, and that it would interact with ANT only under exceptional circumstances. Some data support this. In one study, aged mitochondria were frozen and thawed, and the recovery of CyP-D in the membrane fraction assayed immunologically [44]. Little recovery was observed in the absence of peroxides (thiol oxidation by peroxides is believed to relieve inhibition of PT pore opening by internal adenine nucleotides; section 3.4). This is consistent with CyP-D recruitment by ANT during oxidative stress. By contrast, in another study, purified CyP-D (fusion protein) bound very strongly to detergent-extracted VDAC–ANT irrespective of the presence of adenine nucleotides and of dithiothreitol to maintain reduced thiols [58]. In this case, the association did not seem to depend directly on the oxidation state of the interacting proteins, and a more stable VDAC–ANT–CyP-D complex was indicated. It is possible that other mitochondrial components influence the binding of CyP-D with VDAC–ANT and that an effect of redox state on the association is mediated by another protein.

But how does CSA block the pore? CSA is a cyclic undecapeptide with largely unmodified alkyl side chains, which sits in a hydrophobic pocket in CyP corresponding to the active site. Residues 1, 2, 3, 9, 10 and 11 provide the CyP-binding domain and the remainder (residues 4–8) are exposed to the solvent [76–78]. Modification of CSA residue 4 (methyl-leucine to methylvaline) had no effect on its affinity for the pore or CyP-D [52]. Modification of residue 8 (alanine to dansyl-lysine) did decrease cyclosporin potency as a pore inhibitor, but this was matched by an equivalent decrease in its binding affinity for free CyP-D [52]. Thus CSA blockade of the pore seems to be due simply to occupancy of the active site of CyP-D, i.e. there are no indications that the solvent-exposed residues in CSA allow the CyP-D–CSA complex to bind additional proteins (analogous to the CyP-A–CSA–calcineurin complex [79]).

It is generally assumed that CyP-D associates with ANT via the active site and that CSA blocks the pore by preventing this association. In line with this, the binding of CyP-D (affinity matrix) to ANT in Triton X-100-solubilized membranes (above) was blocked by CSA [59]. The same study reported that the interaction between ANT and CyP-D is also blocked by both bongkrekate and by atractylate; the finding that the atractylate–ANT complex in Triton X-100 does not bind to CyP-D needs to




**Scheme 3 Possible mode of PT pore activation under pathological conditions**

Under conditions associated with oxidative stress, the VDAC–ANT–CyP-D complex binds mitochondrial $Ca^{2+}$ with a changed conformation (*) capable of flickering into an open-pore state.

be reconciled with the observation that atractylate-induced pore opening in intact mitochondria is blocked by CSA [50]. On the other hand the binding of CyP-D to the VDAC–ANT complex was found to be unaffected by CSA, which indicates that the VDAC–ANT–CyP-D–CSA forms a stable complex [58,80] (Interestingly, the binding of CyP-A to Aop1 also occurs in the presence of CSA [72].). According to these latter findings, CSA would not block the pore by preventing the binding of CyP-D to the pore complex. Conceivably, it may block in one of two ways. First, CSA is a reasonable size and it may block solute flux sterically if it binds close to the pore entrance. In agreement with this, once mitochondria have lost internal solutes (adenine nucleotides etc), CSA blocks pore-mediated movement of large solutes, but not $H^+$ [47,56]. Secondly, CSA might induce inhibitory conformational changes. When CSA binds to free CyP, there is very little structural change in the protein [77,81]. But pore opening might bring about changes in CyP-D conformation within the triprotein complex. If the thus-constrained CyP-D bound CSA with decreased affinity, then this could account for CSA inhibition; CSA would displace the equilibrium to the closed state as outlined in Scheme 3. The hypothetical model also offers an explanation for the decrease in CSA binding to CyP-D (in intact mitochondria) in the presence of $Ca^{2+}$ [47,53,54]. By inducing pore opening, $Ca^{2+}$ would shift the distribution to constrained CyP-D with low binding affinity for CSA. Conversely, adenine nucleotides induce pore closure and would be expected, according to the model, to promote CSA binding to CyP-D (in mitochondria), which they do [53,54,56]. But other models could be construed to explain the data. The model outlined (Scheme 3) is simply a basis for future work.

### 2.6 Which component provides the pore?

The diameter of the open PT pore has been estimated to be 2.0–2.6 nm (section 1.3). How do these estimates compare with those obtained with purified VDAC and ANT? Reconstituted VDAC yields two conductance states depending on membrane potential. From measurements of permeability to poly(ethylene glycol)s and γ-cyclodextrin the two states have estimated diameters of 2.5–3.0 nm (low potential) and about 1.8 nm (high potential) [82,83]. Thus the PT pore is about the same size as VDAC, but one cannot attribute the PT pore to either state of VDAC in particular.

Conductance measurements allow further comparisons. These have been made on mitochondrial mitoplasts, in which the outer membrane is largely stripped off by treatment with digitonin, but which retain porin at inner-/outer-membrane contact sites. Patch-clamp measurements have detected a large, 1.2 nS channel (in 150 mM KCl) which displays a 600 pS substate [84,85]. By comparison, the fully open VDAC in planar bilayers yields a conductance equivalent to 600 pS in 150 mM KCl [83]. It has been suggested that the mitochondrial 'megachannel' reflects co-operative behaviour between two VDAC molecules [84]. Purified ANT in planar bilayers is reversibly altered by high [$Ca^{2+}$] to produce a pore with conductance equivalent to 900 pS in 150 mM KCl [41,42]. Like the PT pore, the ANT pore is activated by atractylate and blocked by bongkrekate. Thus conductance measurements are roughly consistent with either ANT or VDAC, or both components, forming the PT pore. But does the 'megachannel' detected in conductance measurements actually reflect the PT pore? On the one hand, the 'megachannel' is activated by $Ca^{2+}$ and blocked by CSA [86,87], two basic properties of the PT pore. However, a 'megachannel' has been detected in mitoplasts prepared from mitochondria of yeast mutants lacking all ANT isoforms [88]; the channel is also insensitive to atractylate. This particular issue is unresolved.

## 3 PT PORE INVOLVEMENT IN NECROTIC CELL DEATH

There are gathering indications that PT pore activation is important in the pathogenesis of necrotic cell death. Most of the data relate to death arising from tissue $Ca^{2+}$ overload and oxidative stress in connection with tissue ischaemia and reperfusion. Ischaemia is associated with impaired energy metabolism culminating in cell death. With 'moderate' periods of ischaemia, the depressed cellular energy state is fully reversed on reperfusion, and cells remain viable and functional. But with prolonged ischaemia, reperfused cells are dysfunctional and may even die, a phenomenon known as 'reperfusion injury'. Reperfusion injury is encountered clinically in by-pass surgery, thrombolysis and organ transplantation. Evidently changes occur during ischaemia that render tissues adversely sensitive to oxygenated blood flow when reintroduced. An understanding of these changes is important for the design of cardioplegic solutions used to maintain the underperfused heart during by-pass surgery and in maintaining donor organs for transplantation. Among the changes that are believed to be critical are the losses of ATP and of adenine nucleotides, and the increases in intracellular $Ca^{2+}$ and $P_i$. But what determines when such changes become irreversible? Can we define a point of no return?

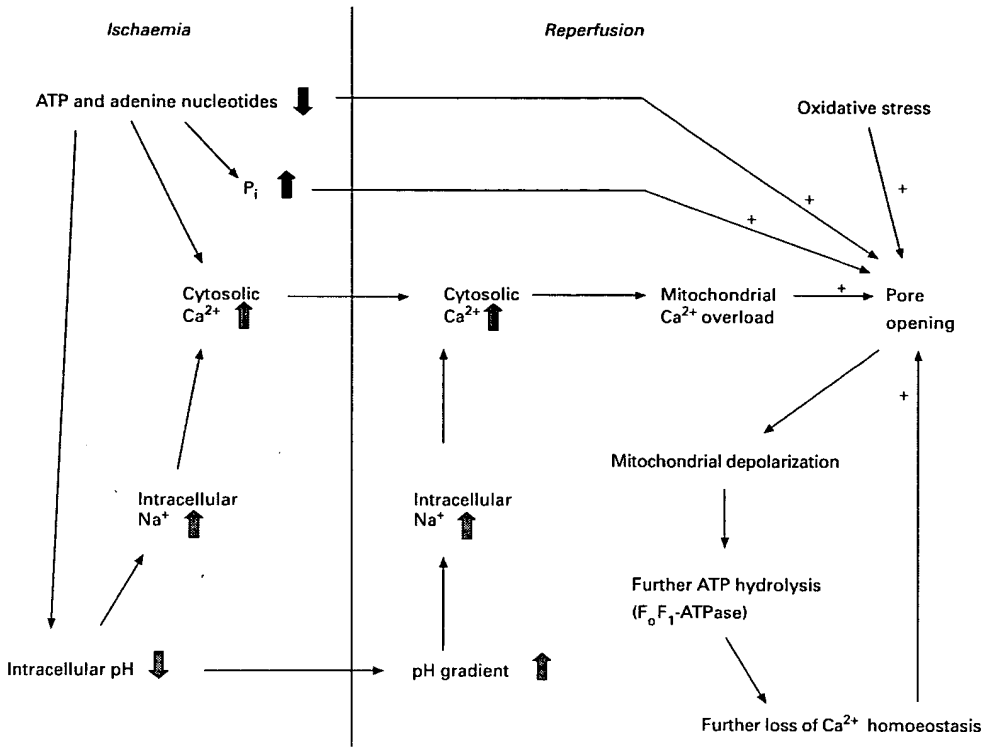
In 1988 I and my co-workers suggested that the PT pore might have a major role in necrotic cell death associated with ischaemia and reperfusion [30,49,89]. As a working hypothesis, we proposed that the changes in $Ca^{2+}$, $P_i$ and adenine nucleotides during ischaemia, together with the oxidant stress arising on reperfusion (sections 3.2–3.4), would trigger PT pore opening. Since PT pore opening leads to mitochondrial ATP hydrolysis, rather than synthesis, energy metabolism would be further impaired, resulting in further $Ca^{2+}$ deregulation, further PT pore opening, and so on. The cell would enter a vicious cycle of decreasing phosphorylation potential and capacity for $Ca^{2+}$ control, leading inevitably to cell death. According to the hypothesis (Scheme 4), PT pore opening would be the critical point at which the injury becomes irreversible [30,49]. At the same time we found that CSA was a potent PT pore inhibitor and suggested that it would provide a means of testing the hypothesis [49]. In agreement, CSA protected against the losses of inner-membrane potential [46], ATP [90,91] and cell viability [90] in cardiomyocytes subjected to anoxia–reoxygenation (reviewed in [4,5,43,91]).

### 3.1 'Hotdogs' with calcein

CSA protection has now been observed in numerous (but not all) situations involving impaired $Ca^{2+}$ metabolism and oxidative stress. These include hepatocytes (oxidative stress [92–94]; anoxia [95]; reoxygenation [96]), endothelial cells (reoxygenation [97]), heart (reperfusion [98]) and brain (transient ischaemia [99]; *N*-methyl-D-aspartate [100]). But as more has become known of CSA and its target proteins, it has become clear that CSA is a far from incisive means of diagnosing specific cellular events. CSA blocks the pore by binding to CyP-D in the mitochondrial matrix (section 2.2). But human cells contain at least eight other CyPs, including enzymes resident in the cytosol (CyP-A, CyP-40), endoplasmic reticulum (CyP-B, CyP-C) and nucleus (CyP-33, CyP-60, Nup350) [101–107]. Thus CSA would affect at least nine intracellular proteins, the functions of which have yet to be fully defined. CyP-B may be involved in $Ca^{2+}$ control in the endoplasmic reticulum [107]. CyP-A activates Aop1, which protects against oxidative stress, although this appears to be CSA-insensitive [72]. In addition, the CyP-A–CSA complex inhibits the $Ca^{2+}$/calmodulin-dependent protein phosphatase calcineurin [79]. Multiple CSA targets are indicated in heart cells, at least, by the fact that CSA protection is expressed only over a narrow range of CSA concentrations, consistent with both protective and 'antiprotective' targets [90,98]. Clearly CSA protection needs to be supplemented by additional measurements of mitochondrial function if PT pore involvement is to be validated. Two such approaches have been used.

Griffiths and Halestrap [108] used a technique applicable to perfused organs. Hearts were perfused with radiolabelled 2-deoxyglucose (DOG) which becomes entrapped in the cytoplasm as DOG 6-phosphate. After extracellular washout, the hearts were homogenized in the presence of EGTA (which closes PT pores quickly). Radiolabel recovery in the mitochondrial fraction was used as an index of PT pore opening before homogenization. The 'hotdog' approach detected PT pore opening on reperfusion when this was associated with tissue injury (the loss of contractile function). Since cells within perfused organs may behave heterogeneously, some dying, others not, it is sometimes difficult to discern the order of events, i.e. whether PT pore opening precedes cell death (and is causative), or occurs afterwards. But DOG entrapment in mitochondria clearly requires that PT pore opening takes place before the plasma membrane becomes permeable to DOG, and is consistent with a causal relation between pore opening and cell death. A recent application of this technique suggests that some PT pore opening on reperfusion may, subsequently, be reversed as reperfusion progresses, implying that tissues can tolerate a small degree of PT pore opening on reperfusion [109]. The topic of transient pore opening is considered in section 4.3.

Lemasters and co-workers employed a technique applicable to single cells. Cells were incubated with a lipid-soluble calcein ester which, after hydrolysis to the free acid in the cytoplasm, becomes entrapped in that compartment. The cells were simultaneously loaded with TMRME, which accumulates electrophoretically in mitochondria and provides an index of the inner-membrane potential. The distribution of the two dyes was imaged using confocal microscopy. In healthy hepatocytes and cardiomyocytes, mitochondria excluded calcein (fluorescence) indefinitely. However, hypoxia or oxidative stress (t-butylhydroperoxide) caused calcein entry into the mitochondrial space concomitant with membrane potential dissipation [110,111]. These events were blocked by CSA [112]. The simultaneous use of calcein and TMRME has been queried on the basis that TMRME quenches calcein fluorescence, so that any calcein




**Scheme 4 Involvement of the PT pore in ischaemia–reperfusion-induced cell death**

ATP dissipation during ischaemia leads to rises in resting cytosolic free [$Ca^{2+}$] and $P_i$. Reperfusion leads to excessive mitochondrial $Ca^{2+}$ uptake. Mitochondrial $Ca^{2+}$ overload together with oxidative stress and the prevailing high $P_i$ and low ATP provoke PT pore opening. This initiates a 'vicious cycle', i.e. inner-membrane depolarization, ATP hydrolysis by the mitochondrial ATP synthase, further increases in cytosolic $Ca^{2+}$, and so on, leading to cell death.

preloaded (via the ester) into mitochondria may only become evident on loss of TMRME [113]. But similar experiments have been conducted with singly loaded cells. When hepatocytes were loaded with dichlorofluorescein, PT pore opening could be detected as a loss of the fluorophore from the mitochondria; this was followed by its release from cells as they lost viability [114]. In addition, protonophoric uncoupling agents collapsed the inner-membrane potential and released TMRME, but did not lead to apparent redistribution of calcein [110].

The case for PT pore involvement in the pathogenesis of necrotic cell death is strengthened by the correlation between the *in vitro* conditions necessary for pore opening and the intracellular conditions that develop during ischaemia–reperfusion (Scheme 4). These correlations are examined in more detail below.

### 3.2 Adenine nucleotides and $P_i$

Ischaemia is associated with a progressive loss of ATP. This is accompanied by only a small temporary rise in ADP as the adenine nucleotides are degraded to nucleosides and bases [115–117]. There is therefore a net loss of adenine nucleotides. Adenine nucleotides inhibit pore opening in isolated mitochondria triggered by either $Ca^{2+}$ and $P_i$ or by $Ca^{2+}$ and oxidative stress. *In vivo*, this protection would be expected to disappear as adenine nucleotides are lost. In addition, the net loss of adenine nucleotides is associated with a massive rise in tissue [$P_i$] to concentrations exceeding 20 mM [118]. $P_i$ co-activates the PT pore with $Ca^{2+}$ [30,33]. Adenine-nucleotide depletion, therefore, leads to both activation and deprotection.

Both ADP and ATP, but not AMP, inhibit the PT pore, but there is no consensus over which is the most effective. In general, assay procedures that involve preopening of the pore show ADP as the most inhibitory (e.g. [44,119]). On the other hand, when adenine nucleotides are used to prevent pore opening, then only ATP is effective [43]. ATP prevention of pore opening necessarily involves its acting on the outside of the mitochondrial inner membrane, whereas inhibition by ADP (after pore opening) could occur by binding to the translocase at the inner face of the inner membrane. The lack of consensus, therefore, could simply reflect different binding sites for the nucleotide under different assay conditions. In any event, ATP prevention is clearly more relevant to deprotection during ischaemia. A concentration of 5 mM ATP, similar to that found physiologically in the cytosol, blocked pore opening in intact heart mitochondria completely, whereas 1.5 mM ATP (buffered enzymically) inhibited only partially [43]. 1 mM ADP (buffered enzymically), a concentration in excess of that attained in ischaemia [116], gave no protection [43]. These relative potencies bear no relation to the binding affinities of ANT for ADP and ATP (ADP affinity > ATP affinity [120]), and suggest that ATP can inhibit the pore by binding to some other component. Possible candidates are the kinases associated with the VDAC–ANT complex (section 2.4) (Scheme 2). From these data it seems that cells may become vulnerable to PT pore opening when rather more than two-thirds of cellular ATP has been dissipated. In this connection, it is noteworthy that the time course of ATP dissipation in global ischaemia is biphasic ($^{31}$P-NMR measurements in heart [115]). The second phase commences at about 65% ATP loss and

follows a pronounced rise in cytosolic $Ca^{2+}$. It is not known, however, whether the second phase is due to pore opening.

### 3.3 $Ca^{2+}$

$Ca^{2+}$ is the fundamental PT pore activator in almost all reports in the literature. To the best of my knowledge the only reported exception is PT pore opening in single immobilized mitochondria induced by intramitochondrially generated reactive oxygen species [37]. Intramitochondrial $Ca^{2+}$ activates the PT pore by binding to low-affinity sites, i.e. $K_d$ 25 $\mu$M [33], increasing to $K_d$ > 200 $\mu$M in the presence of ADP [44]. Since intramitochondrial free $Ca^{2+}$ is normally maintained below 10 $\mu$M, it is clear that severe mitochondrial $Ca^{2+}$ overload is needed for pore activation.

As discussed in section 1.3, the mitochondrial $Ca^{2+}$ cycle would be expected to produce mitochondrial $Ca^{2+}$ overload when basal (resting) cytosolic free [$Ca^{2+}$] rises. Resting cytosolic free [$Ca^{2+}$] increases during ischaemia, and increases further and more abruptly on reperfusion, when this is associated with injury. These changes have been extensively documented, in particular in heart and brain (e.g. [121,122] and references cited therein). Cytosolic free [$Ca^{2+}$] in whole organs, such as heart, can be measured by $^{19}$F-NMR of the $Ca^{2+}$ indicator, 5-fluorobis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid ('5F-BAPTA'; [115]). In perfused hearts the rise in $Ca^{2+}$ begins when about two-thirds of cell ATP has been depleted, and reflects the failure of $Ca^{2+}$ pumps in the plasma membrane and sarcoplasmic reticulum as the cytosolic phosphorylation potential falls. Intracellular acidification (from lactate) also contributes, by leading to increased intracellular $Na^+$ (plasma-membrane $Na^+ \leftrightarrow H^+$ exchange) and, consequently, impaired plasma membrane $Na^+ \leftrightarrow Ca^{2+}$ exchange. This becomes pronounced on reperfusion, when the acidic extracellular fluid is washed out, leading to a high pH gradient across the plasma membrane [123] (Scheme 4). The contribution of altered $Na^+$ gradients to $Ca^{2+}$ overload can be evaluated with amiloride, which inhibits the plasma-membrane $Na^+ \leftrightarrow H^+$ exchange. Amiloride abolished the rise in intracellular $Na^+$ ($^{23}$Na NMR) during ischaemia, and markedly delayed the rise in cytosolic free [$Ca^{2+}$], without affecting the decreases in intracellular pH and ATP ($^{31}$P NMR) [124].

In the early stages, the rise in resting cytosolic free [$Ca^{2+}$] in ischaemia (heart) or anoxia (isolated myocytes) is promptly restored to low physiological levels on reperfusion/reoxygenation [24,115,125]. This indicates that the $Ca^{2+}$ rise precedes cell death. Cobbold and co-workers [125], using cardiomyocytes loaded with aequorin as $Ca^{2+}$ indicator, made the important observation that reoxygenation only restores low resting cytosolic [$Ca^{2+}$] if a critical limit of 1–2 $\mu$M $Ca^{2+}$ is not exceeded. When cytosolic free $Ca^{2+}$ rose above this limit during anoxia, then reoxygenation failed to re-establish $Ca^{2+}$ homoeostasis, and cell death ensued. This limit is remarkably close to the set point, the resting cytosolic [$Ca^{2+}$] that produces mitochondrial $Ca^{2+}$ overload (section 1.3), suggesting that mitochondrial $Ca^{2+}$ overload and PT pore activation may be a precondition of this form of cell death. In broad agreement, application of the Indo 1/$Mn^{2+}$ technique (section 1.1) to whole hearts indicated that the loss of contractile function on reperfusion was related to increased mitochondrial, rather than cytoplasmic, [$Ca^{2+}$] [126].

### 3.4 Oxidative stress

Reperfusion of ischaemic tissue leads to overproduction of superoxide anion ($O_2^-$) and $H_2O_2$. $O_2^-$ can arise from several sources. One source is the reaction catalysed by xanthine oxidase. This enzyme is found in endothelial cells almost entirely in the dehydrogenase form, which utilizes $NAD^+$ as electron acceptor. During ischaemia the enzyme can be converted by a $Ca^{2+}$-dependent protease into the oxidase, which reduces $O_2$ to form $O_2^-$ [127]. Hypoxanthine accumulates in ischaemic tissue as a degradation product of adenine nucleotides, thus providing a high concentration for the oxidase on reoxygenation [116]. The contribution of xanthine oxidase to $O_2^-$ production has been debated (reviewed in [128,129]). Allopurinol, an inhibitor of xanthine oxidase, decreases reperfusion injury in some models, but can also act as free-radical scavenger. Nevertheless, in some studies (e.g. [130]) allopurinol inhibited the reoxygenation-induced increment in $O_2^-$ without affecting basal $O_2^-$ production, consistent with significant production of $O_2^-$ by xanthine oxidase. Another source of reactive oxygen species are neutrophils, which accumulate at the site of injury and release $O_2^-$ via NADPH oxidase. Endothelial cells appear to be the major regulators of neutrophil recruitment via the expression surface adhesion molecules for cell–cell interaction [131]. Similar mechanisms lead to the recruitment of platelets [132], which produce large amounts of $O_2^-$ on reoxygenation [133]. $O_2^-$ is converted into $H_2O_2$ by superoxide dismutase resident in both intracellular and extracellular compartments [128]. Thus reperfusion leads to increased production of $O_2^-$ and $H_2O_2$ at sites external to the parenchymal cells. This allows their interception by superoxide dismutase and catalase added to the perfusion medium, an intervention that yields a degree of protection against irreversible injury [128,129]. Once formed, $H_2O_2$ readily diffuses across cell membranes. Reperfusion also leads to overproduction of $O_2^-$ and $H_2O_2$ by mitochondria of the parenchymal cells. It has been calculated that 2–4 % of the $O_2$ consumed normally by the respiratory chain is incompletely reduced, yielding $O_2^-$ [134]. Immediately on reperfusion this proportion is increased in a burst of $O_2^-$ production, owing to the build up of one-electron donors in the respiratory chain during the preceding anoxic phase [135]. Thus mitochondria are especially vulnerable to oxidative stress on reperfusion.

Reperfusion-induced oxidative stress can be mimicked by addition of peroxides to cells in culture. t-Butylhydroperoxide is frequently used, and causes lethal injury. Addition of t-butylhydroperoxide to hepatocytes leads to oxidation of GSH and pyridine nucleotides through the sequential actions of glutathione peroxidase, glutathione reductase and pyridine nucleotide transhydrogenase [114]. This is followed by PT pore opening (calcein redistribution, section 3.1) and cell death. Peroxides are equally effective in inducing PT pore opening in isolated mitochondria provided that $Ca^{2+}$ is also present; peroxides alone are ineffective [30,89]. In this sort of experiment, $P_i$ can substitute for t-butylhydroperoxide as co-activator with $Ca^{2+}$ [30]. However, Vercesi and co-workers [136] found that high [$P_i$] increases basal $H_2O_2$ production by respiring mitochondria, so again the peroxide requirement may be satisfied. In agreement, $Ca^{2+}$-plus-$P_i$-induced pore opening was partially blocked by added catalase [136]. The requirement for both oxidant stress and $Ca^{2+}$ for PT pore opening in isolated mitochondria implies a similar dual requirement *in vivo*. This question has not been directly addressed, but peroxide-induced oxidative stress is typically associated with cellular $Ca^{2+}$ overload [137]. Tissue reperfusion after prolonged ischaemia is associated with elevated $H_2O_2$ and $Ca^{2+}$ (section 3.3), thereby satisfying the dual conditions for PT pore opening.

Oxidant-stress-induced pore opening is readily reversible. Thus pore opening in isolated mitochondria induced by hyperoxia, and $Ca^{2+}$ is fully reversed on restoration of normoxia [91]. Under these conditions the $O_2^-$ produced by the respiratory chain increases linearly with $O_2$ tension [138] with the formation of $H_2O_2$ (superoxide dismutase). Mitochondria lack catalase, and

the $H_2O_2$ is reduced by GSH (glutathione peroxidase). From this, it appears that pore activation is probably mediated via oxidation of the GSH, NADPH or NADH pools.

Bernardi's group have provided evidence that oxidative stress triggers PT pore opening by oxidizing *vic*-thiols in the pore protein. Arsenite attacks *vic*-thiols and can substitute for peroxides as co-activator with $Ca^{2+}$ [139,140]. Activation by peroxides and by arsenite is blocked by *N*-ethylmaleimide (NEM). This has led to a model in which the linkages –S–S– and –S–As(OH)–S– in the pore structure yield pore opening, whereas $(–SH)_2$ and –SH,–S(NEM) produce pore closure. According to this model, the pore is maintained in the closed form by the normally highly reduced state of intramitochondrial glutathione. Oxidation of the GSH pool during excess production of peroxides allows dithiol formation and pore opening.

The use of a phenylarsine affinity matrix has tentatively identified ANT as the constituent containing the relevant *vic*-thiols [44]. The same group investigated the role of the thiols using an assay system first introduced by Haworth and Hunter [141], which allows replacement of internal solutes. The capacity of internal adenine nucleotides to inhibit the PT pore was decreased by thiol oxidants, suggesting that thiol oxidants activate by preventing the binding of inhibitory adenine nucleotides at the m-face of the inner membrane. This agrees broadly with the properties of the PT pore reconstituted from the VDAC–ANT–CyP-D complex, which yielded $Ca^{2+}$-induced PT pores in the absence of thiol oxidants [58]. Thus oxidative stress may activate the PT pore by overriding inhibition by intramitochondrial adenine nucleotides.

### 3.5 The inner-membrane potential

During ischaemia, when electron transport ceases, the inner-membrane potential is developed at the expense of ATP hydrolysis by the mitochondrial ATP synthase. In $CN^-$-poisoned myocytes, for example, the decay of the membrane potential (rhodamine fluorescence) was greatly accelerated when mitochondrial ATP hydrolysis was blocked by oligomycin [46]. Using single cardiomyocytes loaded with fluorescent indicators of membrane potential (JC-1) and ATP (indirectly; magnesium green), Leyssens et al. [142] observed a close correspondence between ATP dissipation and membrane potential decay when anaerobic and aerobic catabolism was blocked ($CN^-$ plus DOG). Mitochondrial depolarization may facilitate pore opening. Thus in isolated mitochondria titrated with uncoupling agents to manipulate the membrane potential, PT pore opening increased with increased depolarization [143–145]. There does not appear to be a critical potential, rather an increasing propensity for pore opening as the mean membrane potential of the population falls (but a critical potential would probably not be evident in experiments using populations, since mitochondria would be out of phase). The observed range over which changes in potential affect the PT pore, 180–120 mV, would not be expected to lead to losses of mitochondrial $Ca^{2+}$ (section 1.1).

It is not yet established whether the uncoupler-induced activation of the pore signifies a potential-sensitive component of the PT pore complex. The $Ca^{2+}$-induced pore activity of purified ANT (section 2.1) shows a similar dependence on potential, suggesting that the voltage-dependency resides in ANT. On the other hand, uncoupling of the respiratory chain, used to manipulate membrane potential (above), leads to increased $O_2^-$ production [136]. Experiments employing uncoupling agents at various $O_2$ tensions, thereby varying $O_2^-$ formation, have yielded conflicting results, suggesting a major [146] or minor [147] contribution of oxidative stress to uncoupler-induced pore activation. It is also possible that the energy-linked transhydrogenase provides a link between potential and pore activity. Depolarization would decrease the capacity of the energy-linked transhydrogenase to maintain a high NADPH/NADP ratio; as a result, the GSH/GSSG ratio would also decrease, allowing *vic*-thiol oxidation.

Mitochondrial depolarization *per se* is insufficient to produce PT pore opening *in vivo*. Thus treatment of hepatocytes with uncoupling agents does not lead immediately to PT pore opening (calcein entry; section 3.1) [110]. Under these circumstances, PT pore opening presumably requires loss of ATP and high cytosolic $[Ca^{2+}]$, in line with the general properties of the pore.

### 3.6 Cellular acidosis

Ischaemia is associated with intracellular acidosis as a result of increased lactic acid production and lack of tissue washout. For example, during global ischaemia the intracellular pH of rat heart fell from 7.1 to 6.5 in 20 min [124]. Work with isolated mitochondria shows that such a shift in pH depresses pore opening [33,148]. This is frequently taken to be a protective aspect of tissue acidosis (e.g. [38,109]). However, the issue is really more complex, since the pH-dependency of $H^+$ flux via open pores should also be considered. Pore-mediated $H^+$ backflow across the inner membrane would be given by

$$\text{Rate of } H^+ \text{ backflow} = C[H^+] \text{ (proton electrochemical gradient)}$$

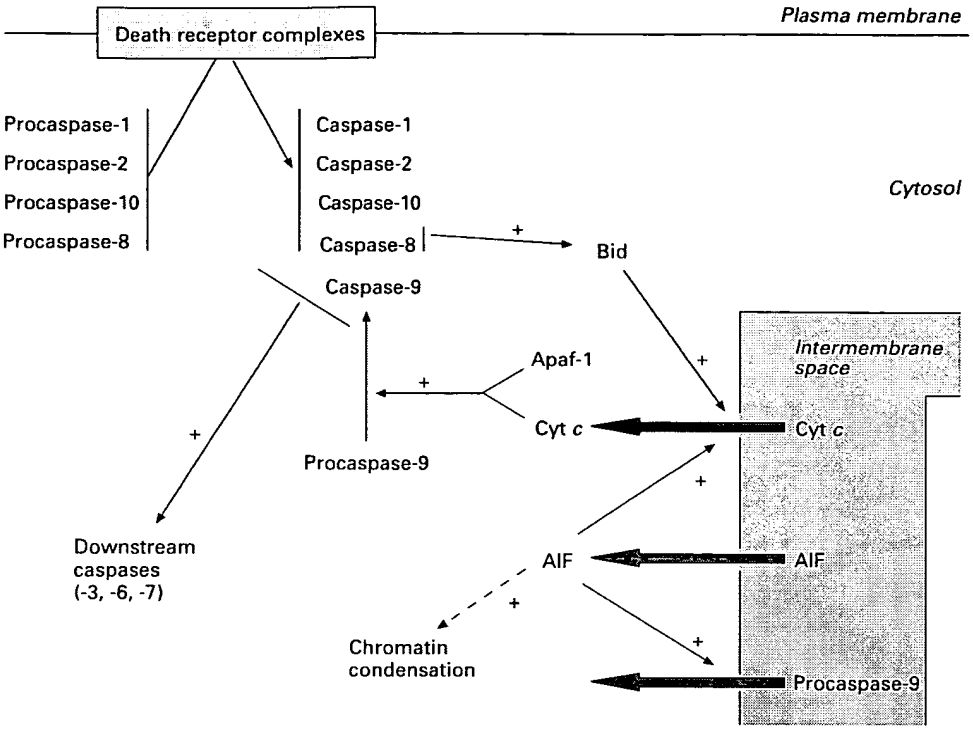
Where $C$ comprises the number (fractional) of open pores and their $H^+$ conductance. Thus decreased pore opening (flicker) with decreased pH would be offset by increased $[H^+]$. In practice pore opening still occurs at pH 6.5, and the rate of membrane-potential collapse, a function of $H^+$ backflow, proceeds more quickly [33]. Thus tissue acidification might even exacerbate pore-induced injury.

## 4 THE INVOLVEMENT OF THE PT PORE IN APOPTOSIS

Cellular breakdown during apoptosis is executed by caspases, a family of ten or more cysteine proteinases active at aspartic acid residues (reviewed in [149]). Caspases are expressed constitutively as inactive proenzymes and become activated after proteolyic cleavage. Caspases-3, -6, and -7 (class II caspases) contain short pro-domains and are believed to be activated by other (class I) caspases. Class I caspases possess long N-terminal pro-domains, and are self or mutually cleaved after aggregation into complexes. Aggregation is brought about by adaptor proteins that interact with the extended pro-domains. Two types of caspase interaction domain have been recognized. Procaspases-8 and -10 are recruited by the adaptor FADD (Fas-activated protein with death domain) within activated death receptor complexes at the plasma membrane (death-inducing signalling complex, DISC). FADD contains a death effector domain (DED) which interacts with DEDs in the pro-domain of caspases-8 and -10. Other caspases are recruited via a CARD (caspase recruitment domain). Procaspases-1 and -2 appear to be recruited, respectively, into the cell-surface death receptor complexes via the CARD-carrying adaptor proteins CARDIAK and RAIDD. In the case of procaspase-9, the CARD-carrying adaptor protein has been identified as Apaf-1 [150] (Scheme 5).

### 4.1 The mitochondrial connection

In 1996, Liu et al. [151] made the critical observation that caspase activation by Apaf-1 in a cell-free system required dATP and




**Scheme 5 Involvement of mitochondria in apoptosis**

The activation of class 1 caspases at plasma-membrane death receptor complexes (DISC, pink) leads in turn to activation of downstream caspases. Caspase activation of Bid leads to recruitment of key apoptogenic proteins from the mitochondrial intermembrane space. The release of these intermembrane-space (grey) proteins is also promoted by Bax (not shown). Abbreviation: Cyt *c*, cytochrome *c*.

cytochrome *c* (Scheme 5). Cytochrome *c* binds to Apaf-1, possibly at its C-terminal end, since a C-terminally truncated form of Apaf-1 no longer requires the cytochrome [152]. The complex self-associates and recruits procaspases, which are then processed to their active forms. These include caspases-4, -8 and -9, the latter being recruited most strongly [153]. The requirement of cytochrome *c* by an apoptotic pathway was the first incontrovertible evidence for the involvement of mitochondria in apoptosis. Subsequent work revealed that cytochrome *c* translocates from mitochondria into the cytosol just a few hours into the apoptotic programme, e.g. after stimulation with Fas ligand, tumour necrosis factor, staurosporine or withdrawal of growth factor ([154,155] and references cited therein).

Like most mitochondrial proteins, cytochrome *c* is encoded in the nucleus. Apo-cytochrome *c* synthesized in the cytoplasm is imported in an unfolded state into the mitochondrial intermembrane space; here, the haem group is covalently attached, and the holoenzyme assumes its mature conformation. Apocytochrome *c* is apoptotically inactive. Cytochrome *c* is the sole water-soluble cytochrome and acts as a mobile carrier of electrons between the bc1 complex and cytochrome oxidase. It binds electrostatically to negatively charged surfaces of these complexes at the outer face of the inner membrane [156]. Since electrons flow rapidly down the respiratory chain, cytochrome *c* can associate and dissociate rapidly with each complex, and is not tightly bound to either. It is normally restricted to the intermembrane space by the integrity of the outer membrane.

How are mitochondria persuaded to loose cytochrome *c*? The key appears to be Bid, a 26000-$M_r$ protein resident in the cytosol. Wang's group showed that Bid is cleaved by caspase-8 *in vitro* to produce a 15000-$M_r$ C-terminal fragment which binds to isolated mitochondria and brings about the release of cytochrome *c* [155]. Bid cleavage occurs *in vivo* early in apoptosis [157]. It appears, therefore, that caspase-8 activation at the DISC can lead to Bid cleavage and release of cytochrome *c* to the cytosol (Scheme 5).

But why do cells enlist the help of mitochondria to execute apoptosis? A clue to this emerges from the fact that mitochondrial involvement seems to depend on stimulus and cell type. Yoshida et al. [158] broached this question using Apaf-1 knockout mice. When different cell types were examined, some apoptotic pathways were functional in the knockouts, others not. Knockout thymocytes, for example, responded normally to fas ligand, indicating that cytochrome *c* release was not an essential part of the fas-mediated pathway in these cells. But the same thymocytes became resistant to a number of other apoptotic stimuli, e.g. dexamethasone, etoposide, irradiation and (less marked) staurosporine. These pathways seems to require Apaf-1 and, by extension, cytochrome *c* release. Scaffidi et al. [159] went on to correlate mitochondrial involvement in fas-generated signals with the efficiency of caspase-8 activation at the DISC. In some cells, receptor activation led to caspase-8 activation within seconds and caspase-3 activation after 30 min. In these cells, producing rapid activation of caspase-8 at the DISC, mitochondrial engagement was not essential (although it did occur later in the programme, as evidenced by cytochrome *c* release). However, in other cell types gross activation of both caspases

was delayed for 1 h. Here, it seems, minimal activation of caspase-8 occurred at the DISC, and mitochondrial cytochrome *c* release was used as a signal amplifier. Signal amplification by the mitochondrial 'detour' added about 1 h to the apoptotic programme.

Mitochondrial involvement in apoptosis has recently been consolidated by the finding of other major pro-apoptotic factors in the intermembrane space. These comprise a proportion of certain procaspases, including procaspase-9 [160,161], and a 57000-$M_r$ apoptosis-inducing factor, AIF [162,163] (Scheme 5). Kroemer's group have identified AIF as flavoprotein showing sequence similarity to bacterial ferredoxin/NADH oxidoreductases, but its role as an oxidoreductase is obscure [164]. A number of key observations establish its importance in apoptosis. Thus, in normal cells, AIF is restricted to mitochondria (immunofluorescence studies), but induction of apoptosis leads to AIF translocation to the cytosol and to the nucleus [164]. When it was added to isolated nuclei, AIF brought about chromatin condensation and DNA cleavage into large fragments [164]. Recombinant AIF, without the flavin prosthetic group, was similarly active. When injected into the cytoplasm of cells, AIF induced nuclear chromatin condensation; it also caused exposure of phosphatidylserine on the outside of the plasma membrane, a feature of apoptosing cells [164]. When added to isolated mitochondria, AIF induced the release of cytochrome *c* and caspase-9. The latter finding suggests the possibility of a positive feedback loop [164], as illustrated in Scheme 5. Thus, like cytochrome *c*, AIF seems to be bifunctional, with both oxidoreductase and apoptogenic functions.

Bid belongs to the Bcl-2 family of proteins, of which a dozen have been recognized. Some of these promote apoptosis, e.g. Bid, Bax, whereas others, e.g. Bcl-2, inhibit. Bcl-2 is resident in the mitochondrial outer membrane, along with the endoplasmic reticulum and nucleus [165]. Bax is a cytosolic protein, but translocates to mitochondria when the cell receives a death signal [166,167]. The translocation is caspase-mediated, and is believed to entail insertion into the outer membrane via a C-terminal transmembrane domain ([167,168] and references cited therein). Bax and Bcl-2, and also truncated Bid, can heterodimerize via their BH3 domains, and appear to do so in mitochondria. This was elegantly demonstrated by Herman and co-workers [169], who co-expressed the fusion proteins Bax/green fluorescent protein and Bcl-2/blue fluorescent protein and detected tight interactions between the two in the mitochondria of the transfected cells by confocal microscopy and fluorescence resonance energy transfer (a cytochrome *c* fusion did not interact). It seems then that pro-apoptotic Bid and Bax may be recruited to the mitochondrial outer membrane during apoptosis, where they can interact with resident anti-apoptotic Bcl-2. The release of apoptogenic proteins from the intermembrane space may be determined by the relative amounts of Bid, Bax and Bcl-2 in the outer membrane. The way in which these proteins control the release of cytochrome *c* and AIF is an ongoing issue. Bax contains structural similarities to the pore-forming domains of the bacterial colicins and diphtheria toxin [170], and forms ion channels in lipid bilayers [171]. The Bax channel is sufficiently large to admit carboxyfluorescein, and is blocked by interaction with Bcl-2 [171]. But whether Bax forms pores large enough to admit fully folded apoptogenic proteins is another question altogether. Bax added to isolated mitochondria does induce cytochrome *c* release [172]; but truncated Bid, which has no channel-forming domain, is even more effective in this regard [150]. At present therefore, there is no direct evidence for protein-formed pores in the outer membrane large enough to provide a conduit for the release of apoptogenic proteins of the intermembrane space.

### 4.2 Is the pore on the programme?

Breaks in the mitochondrial outer membrane are evident in electron micrographs of Jurkat cells undergoing apoptosis, where they are accompanied by matrix swelling [173]. Are the breaks a result of PT pore activation? In isolated mitochondria PT pore opening is associated with expansion of the matrix space and, since the surface area of the highly folded inner membrane is much greater than that of the outer, this can lead to rupture of the outer membrane and release of apoptogenic proteins from the intermembrane space. For example, when HeLa nuclei were mixed with isolated mitochondria, a range of PT pore activators (atractylate, peroxides, $Ca^{2+}$, diamide) produced mitochondrial swelling, release of cytochrome *c* and AIF, and apoptotic changes in nuclear morphology [163,174–176]. Matrix swelling also precedes delayed neuronal death (hippocampus) triggered by hypoglycaemia, and both the swelling and cell death are prevented by CSA [177]. Accordingly, Kroemer's group have proposed that PT pore opening, leading to matrix expansion and outer-membrane rupture, is the root mechanism for the release of intermembrane-space apoptogenic proteins. In line with this, the PT pore inhibitor bongkrekate not only blocked nuclear apoptosis in this cell-free system, but also blocked dexamethasone-induced apoptosis in thymocytes [174] (in which cytochrome *c* release is an intermediate step; section 4.1).

Kroemer's group also suggest a close functional and physical link between the PT pore and Bcl-2 and Bax in the outer membrane, in theory allowing these proteins to control PT pore activity. In support of this, mitochondria isolated from Bcl-2-transfected cells are more resistant to atractylate- and peroxide-induced pore opening [162]. Bcl-2 overexpression also inhibits nuclear apoptosis in a cell-free system under the same conditions. Conversely, microinjection of fibroblasts with Bax caused mitochondria depolarization, which was blocked by bongkrekate and, therefore, is ascribable to PT pore opening [178]. Thus both pro- and anti-apoptotic Bcl-2 family proteins may interact with components of the PT pore, most logically with VDAC in the outer membrane. There are a number of positive indications for such an interaction. Co-immunoprecipitation studies show that Bax binds to VDAC [179]. When VDAC–ANT preparations containing Bax (along with other mitochondrial proteins), but not Bcl-2, were incorporated into liposomes, PT pore activity (induced by atractylate, $Ca^{2+}$ or peroxides) was blocked when the complex was co-reconstituted with Bcl-2 [178]. Immunodepletion of Bax from the complex led to a loss of atractylate-induced pore activity, but not that induced by peroxides [178]. The use of mitoplasts has provided further evidence. In mitoplasts the outer membrane is very largely stripped off, but some VDAC is retained at residual inner/outer membrane contact sites, where it is presumably complexed to ANT. Mitoplasts bound added Bax and Bcl-2, but the strength of interaction was decreased by CSA [178]. Since CSA targets the VDAC–ANT–CyP-D complex (section 3.2), this complex presumably provided the binding site for Bax and Bcl-2. The model which emerges, therefore, is that Bax and, possibly, Bcl-2 may be recruited to the VDAC–ANT–CyP-D complex that forms the pore (Scheme 2). These complexes form at the outer/inner membrane contact sites, and there are reports that Bcl-2 congregates at these sites [180].

Since PT pore opening depolarizes the inner membrane, measurements of the inner-membrane potential should provide a means of assessing PT pore involvement. Indeed, it has been widely observed that mitochondrial depolarization does occur several hours into the apoptotic programme. The depolarization occurs progressively so that, 1 h or so after the first-detected depolarization, all mitochondria are depolarized. Lemasters and

co-workers [181] followed the intracellular redistributions of TMRME (from the mitochondria) and calcein (from the cytosol), as outlined in section 3.1. They observed that, 8 h after stimulation with tumour necrosis factor, individual mitochondria began to admit calcein and loose TMRME, indicative of PT pore opening. PT pore opening was blocked by CSA, which also inhibited apoptosis in this system. However, in many systems, at least, mitochondrial depolarization occurs downstream of Apaf-1 activation and cytochrome *c* release [158,173,182]. In thymocytes, for example, Apaf-1 knockout not only inhibited caspase-3 activation and apoptosis under dexamethasone or staurosporine, but also abolished the early collapse in inner-membrane potential [158]. Conversely, caspase-3 cleavage and apoptosis under Fas activation proceeded normally in Apaf-1-deficient thymocytes (the apoptotic pathway largely by-passes mitochondria; section 4.1), but the early membrane-potential collapse occurred nevertheless. In addition, active truncated Bid induces cytochrome *c* release *in vitro* without loss of membrane potential [155]. Thus the permanent decay of membrane potential seen in many systems seems to be a consequence of downstream caspase activation, rather than an event leading to their activation. Indeed, it seems rather improbable that PT-pore-induced collapse of the inner-membrane potential would occur in early apoptosis, since it would lead to rapid dissipation of cellular ATP and, as discussed below (section 4.3), cellular ATP seems to be maintained until cytochrome *c* and AIF are released. Of course, transient mitochondrial depolarizations, out of phase between cells, or between mitochondria in a single cell, would go undetected in potential measurements on cell populations. By extension, transient pore opening would also go undetected in experiments of this sort. However, transient pore opening would be evident in experiments of the kind carried out by Lemaster's group [181], in which the intracellular distribution of calcein was imaged. Here, any transient opening would be expected to admit calcein into the mitochondrial matrix, but allow long-term maintenance of inner-membrane potential. But, as discussed above, this behaviour was not observed. At present, therefore, there seems to be no unequivocal evidence that PT pore activation provides a mechanism for outer-membrane rupture under physiological stimuli of apoptosis.

Nevertheless, there is an extensive array of evidence, outlined above, that Bcl-2 and Bax, regulatory proteins of the apoptotic pathway, do markedly influence PT pore formation in response to non-physiological and pathological stimuli (e.g. oxidative stress, atractylate) and that they do so in line with their effects on apoptosis (Bcl-2, inhibitory; Bax, stimulatory). There is also evidence (above) that Bax binds to VDAC. From this it appears that the VDAC–ANT–CyP-D complex can interact with these apoptotic proteins and, by extension, that it may well have an apoptotic role in the normal physiology of the cell. It seems improbable, however, that any such role involves the complex acting as a pore. As outlined in section 3, the PT pore behaviour of the complex seems likely to be expressed only in pathological states (high $Ca^{2+}$, oxidative stress). But PT pore formation by the complex under such conditions could still be influenced by Bcl-2 family proteins interacting with the complex. The following section, therefore, considers the possible role of the PT pore in apoptosis triggered by pathological stimuli.

### 4.3 PT pore opening during 'accidental' apoptosis

Ischaemia, hypoxia, $Ca^{2+}$ overload and oxidative stress are pathological insults leading to necrotic cell death. But the same insults can also cause apoptosis. In experimental models of stroke and myocardial infarction, brought on by a period of ischaemia, the ischaemic core of the tissue undergoes necrotic cell death, but the initially surviving cells in the surrounding regions that have been less severely compromised die subsequently by apoptosis [183–185]. Neurons exposed to massive $Ca^{2+}$ overload become necrotic, but with moderate $Ca^{2+}$ overload they undergo delayed death by apoptosis [186,187]. Similarly, the form of cell death resulting from oxidative stress depends on the intensity of the insult, changing from apoptotic to necrotic as the degree increases [188]. Thus 'mild' forms of these pathological insults evidently engage the apoptotic programme at some point. Might that point be the PT pore? The following addresses this question.

Necrotic cell death is associated with an early loss of ATP, whereas ATP is maintained in the early stages of apoptosis. For example, in staurosporine-treated cells, ATP did not detectably decline until 4 h into apoptosis, by which time cytochrome *c* release to the cytosol was complete [182]. A high cellular phosphorylation potential prevents loss of ionic (e.g. $Ca^{2+}$) homoeostasis and cellular lapse into necrotic cell death. Moreover, ATP is actually needed to execute the apoptotic programme. ATP is required by the ATPase Apaf-1 (section 4.1). In cell-free systems of apoptosis, comprising isolated nuclei and lysates from stimulated cells (e.g. Fas-ligand-stimulated Jurkat cells), added ATP is required for nuclear condensation and fragmentation [189]. The ATP requirement has also been demonstrated with intact cells. Leist et al. [190] manipulated the ATP levels of T-lymphocytes by varying extracellular [glucose]. Exposure to Fas ligand or staurosporine induced apoptotic cell death provided that high intracellular ATP was maintained for 90 min after cell stimulation. If ATP was not maintained, the cells died nevertheless, but without the nuclear morphological changes characteristic of apoptosis. From these observations, if pathological insults such as ischaemia or oxidative stress do bring about apoptosis via PT pore activation, then PT pore opening would need to occur without its causing major ATP depletion. In principle, there are two ways in which this could take place. Mitochondria contain a natural protein inhibitor of the $F_oF_1$-ATPase, termed the 'Inhibitor Protein' [191]. *In vitro*, the Inhibitor Protein limits ATP hydrolysis when the inner-membrane potential is collapsed, but does not interfere with ATP synthesis when the potential is high. The true role of this protein is quite obscure, and it remains possible that it may limit ATP hydrolysis under conditions leading to apoptosis. Secondly, PT pore opening need have little effect on the ATP economy of the cell if it occurred transiently so that the cell was exposed to low inner-membrane potential for brief intervals. If transient PT pore opening were also highly localized, i.e. confined to a few mitochondria, then the impact on the energetic state of the cell would be minimal.

Localized mitochondrial effects are most likely to be caused by localized changes in cytosolic [$Ca^{2+}$]. Measurement of mitochondrial $Ca^{2+}$ using targetted photoproteins has revealed that agonist-induced release of $Ca^{2+}$ from endoplasmic reticulum can elicit large changes in mitochondrial $Ca^{2+}$. It appears that this reflects how close mitochondria are to the intracellular sites of $Ca^{2+}$ release. Pozzan's group, in particular, have provided evidence for the existence of microdomains between closely apposed mitochondria and endoplasmic reticulum, within which there is a close coupling between the release of $Ca^{2+}$ from the reticulum and its uptake by the mitochondria [192–195]. In one study, a three-dimensional image was generated of the mitochondrial and reticular spaces in HeLa cells by loading these compartments with organelle-targetted fluorescent proteins [195]. The compartments showed a high degree of plasticity, but 5–20% of the mitochondrial space was judged to be in very close proximity to the reticulum at any point in time. Digital imaging

of $Ca^{2+}$ release from endoplasmic reticulum by other workers has revealed the elementary events as transient microdomains ('sparks') of high cytosolic [$Ca^{2+}$] [196]. These can trigger the propagation of $Ca^{2+}$ waves across the cell ($Ca^{2+}$-induced $Ca^{2+}$ release), but are often seen as individual events that remain highly localized. Such localized changes in cytosolic $Ca^{2+}$ would produce correspondingly localized, rapid changes in mitochondrial $Ca^{2+}$ within that domain.

Can localized changes in cytosolic [$Ca^{2+}$] trigger the PT pore when accompanied by oxidant stress? Duchen et al. [197] used TMRME to image the inner-membrane potential of individual mitochondria in cardiomyocytes. TMRME accumulates electrophoretically into mitochondria according to the magnitude of the potential, but also photobleaches to generate reactive oxygen species in the matrix compartment. A few mitochondria were seen to undergo occasional, transient depolarizations (flickering), lasting a few seconds, reminiscent of the transient depolarizations in single immobilized mitochondria described in section 1.3. The flickering could be prevented by pre-depleting the sarcoplasmic reticulum of $Ca^{2+}$ (thapsigargin), by blocking $Ca^{2+}$ release from the reticulum (ryanodine), and by blocking mitochondrial $Ca^{2+}$ uptake (DAPPAC). This all indicates that the transiently depolarized mitochondria were in close proximity to the $Ca^{2+}$-release channels of the sarcoplasmic reticulum. Flickering was also blocked by CSA, suggesting that transient pore opening was the cause of it [198], and consistent with the synergistic induction of the PT pore by $Ca^{2+}$ and oxidative stress (sections 3.3 and 3.4). It has been proposed that the juxtapositioning of some mitochondria with the $Ca^{2+}$-release channels of endoplasmic reticulum may produce PT pore opening under physiological circumstances. Thus Jouaville et al. [199] described an intriguing phenomenon in which mitochondrial metabolism clearly influences the propagation of $Ca^{2+}$ waves in oocytes. Waves were induced by injection of Ins(1,4,5)$P_3$. Further injection of substrates for mitochondrial oxidation produced marked changes in periodicity (decreased) and magnitude (increased) of the waves. The changes were abolished by respiratory-chain inhibitors, but restored by injection of substrates for the respiratory chain beyond the block. The same group [35] suggested that mitochondrial $Ca^{2+}$-induced $Ca^{2+}$ release is responsible for the phenomenon (excessive, rapid $Ca^{2+}$ uptake, $Ca^{2+}$-induced PT pore opening, $Ca^{2+}$ release). However, conditions used to demonstrate mitochondrial $Ca^{2+}$-induced $Ca^{2+}$ release *in vitro* [35] amounted to pseudopathological and, as argued in this review, all indications are that PT pore opening requires pathological conditions, e.g. oxidative stress, as in the 'flickering' described above.

Since the mitochondrial intermembrane space is a reservoir of apoptogenic proteins, it follows that the integrity of the outer membrane is probably of paramount importance in preventing 'accidental' apoptosis. Under pathological conditions associated with oxidative stress, however, it seems possible that endo-(sarco)plasmic-reticulum-juxtaposed mitochondria in a small region of the cell may undergo transient PT pore activation and that this could lead to matrix expansion sufficient for outer membrane rupture. If this occurred, then it would be expected to produce catastrophic consequences, sparking a caspase activation cascade, amplified by positive feedback from AIF (Scheme 5). Broadly consistent with this, there are now several reports of cytochrome *c* release into the cytosol after ischaemia and anoxia (e.g. [200]). Although there is clearly much work to be done, it is conceivable that such events may unfold in the intermediate regions of damaged tissue surrounding the necrotic core, where cellular ATP is depleted sufficiently to allow PT pore activation, but insufficiently to compromise the apoptotic pathway. With increased intensity of insult, these basic events could produce necrotic cell death; in this case, the increases in cytosolic $Ca^{2+}$ would become globalized, leading to widespread PT pore opening, inner-membrane depolarization, and loss of the cellular capacity to maintain ATP. The cell would then enter the 'vicious cycle' leading to necrotic cell death as outlined in Scheme 4. But, with lesser insults, localized release of apoptogenic proteins in a small region of the cell may be enough to engage the apoptoptic pathway.

## 5 CONCLUDING REMARKS

A striking aspect of the PT pore complex is that it is assembled from components that have other well-established roles in the life of the cell. The general function of VDAC is to allow low-$M_r$ solute access to the solute-specific transport systems of the inner membrane. ANT mediates ADP $\leftrightarrow$ ATP exchange, essential for the basic bioenergetic function of the organelle. The function(s) of CyP-D is not established, but a likely role is the catalysis of protein folding. Yet, from reconstitutions conducted in different laboratories, it seems that these components assemble into a complex quite readily. In particular there appears to be a native affinity between CyP-D and ANT. This affinity would not interfere with the bioenergetic function of ANT, which is in large excess over CyP-D (e.g. 20-fold in heart mitochondria). The picture which emerges, then, is that the VDAC–ANT–CyP-D complex can exist as a stable entity in the cell under normal physiological conditions and, by extension, that it is assembled for a definite function.

One function of the VDAC–ANT–CyP-D complex is the establishment of contact sites between the inner and outer membranes. The role of contact sites in energy transduction has already been mentioned (section 2.4). In steroidogenic cells the contact sites have a further role in facilitating the transfer of extramitochondrial cholesterol to the inner membrane, where it is converted into pregnenolone [201]. The transfer is controlled by an 18000-$M_r$ benzodiazepine receptor [201] associated with VDAC [202] (Scheme 2). More generally, there are indications that the contact sites are also involved in the transfer of phosphatidylserine between the endoplasmic reticulum and the mitochondrial inner membrane [203,204]. In order to carry out these tasks the sites recruit other proteins, e.g. kinases, benzodiazepine receptor, possibly even endoplasmic reticulum [64], for efficient phospholipid transfer. There is evidence (section 4.2) that the sites also recruit Bax and, possibly, other Bcl-2 family proteins. Thus the VDAC–ANT complex is emerging as a multifunctional recruitment centre for other proteins, assembling these into the appropriate complexes depending on the job at hand. In the case of apoptosis, it appears that the task involves lysis of the outer membrane. It may be that as yet unrecognized proteins are recruited by the complex for outer membrane lysis. Potentially, these could include phospholipases for outer membrane destabilization. It seems important, then, to identify the proteins that form a working partnership with the VDAC–ANT–CyP-D complex during apoptosis.

The VDAC–ANT–CyP-D complex also forms the PT pore, at least under pathological conditions. But whether this provides a physiological mechanism for outer-membrane rupture during apoptosis is currently open to question in view of the repercussions for cellular energy transduction. In my opinion, a possible physiological function of PT pores may be to establish contact between mitochondria in the formation of mitochondrial networks. Skulachev and co-workers have reported evidence that mitochondria *in situ* can form tight intermitochondrial junctions, providing continuity between the matrix spaces of the apposed mitochondria, and allowing the thus-conjugated mitochondria

to operate as a bioenergetic continuum [205]. In this way potential energy in the form of the proton electrochemical gradient might be 'wired' along conjugated mitochondria, permitting efficient energy transfer between different parts of the cell. The concept is quite consistent with the recent three-dimensional reconstructions of the mitochondrial space (section 4.3), which revealed the organelle in HeLa cells as a long, branching tubular structure with marked plasticity, suggesting the possibility that junctions form reversibly according to cellular need. Immunogold studies show that areas of contact between mitochondria are enriched in VDAC [206]. On these grounds it is conceivable that inter-mitochondrial junctions form from PT pores interlocked via VDAC molecules in adjacent mitochondria. Each VDAC in turn would communicate with its own matrix space via interaction with ANT. According to this hypothesis, the VDAC–ANT–CyP-D complex in unconjugated mitochondria would not form a PT pore (i.e. would remain 'closed') under any normal physiological condition. The complex would only form an open PT pore when interlocked with an adjacent PT pore. The physiological mechanism for PT pore opening would thus derive from VDAC–VDAC interactions. With this restriction, physiological PT pore opening would not cause energy dissipation, merely the sharing of the free energy of the proton electrochemical gradient between conjugated mitochondria.

PT pore opening in isolated mitochondria, leading to free diffusion of solutes between the matrix space and the suspending medium, requires pseudopathological conditions of high $Ca^{2+}$, low ATP and an oxidized redox state. These conditions override the normal constraints that keep the pore closed. They also correspond with the cellular changes that unfold during ischaemia/reperfusion. It has been appreciated for decades that $Ca^{2+}$ is instrumental in ischaemic cell death, from the early finding of a link between cellular $Ca^{2+}$ overload and myocardial injury [207] to the more recent extension of the $Ca^{2+}$ model to glutamate-induced delayed neuronal death [208]. But cellular $Ca^{2+}$ overload itself is frequently innocuous. The PT-pore hypothesis, therefore, brings significance to the associated cellular changes in adenine nucleotides and redox state (Scheme 4). At the same time, the hypothesis identifies potential loci for pharmacological interventions against variants of ischaemia-related disease which, collectively, are the major causes of morbidity and mortality in the Western World.

My own work referred to in this article has been supported financially by the British Heart Foundation, the Wellcome Trust and the Medical Research Council.

## REFERENCES


1 Crompton, M. and Heid, I. (1978) Eur. J. Biochem. **91**, 599–608
2 Crompton, M., Capano, M. and Carafoli, E. (1976) Eur. J. Biochem. **69**, 453–462
3 Crompton, M., Kunzi, M. and Carafoli, E. (1977) Eur. J. Biochem. **79**, 549–558
4 Crompton, M. (1990) in Calcium and the Heart (Langer, G. A., ed.), pp. 167–198, Raven Press, New York
5 Crompton, M. (1990) in Intracellular Calcium Regulation (Bronner, F., ed.), pp. 181–210, Wiley–Liss, New York
6 Nicholls, D. G. and Crompton, M. (1980) FEBS Lett. **111**, 261–268
7 Crompton, M., Moser, R., Luedi, H. and Carafoli, E. (1978) Eur. J. Biochem. **82**, 25–31
8 Crompton, M., Heid, I., Baschera, C. and Carafoli, E. (1979) FEBS Lett. **104**, 352–354
9 Goldstone, T. P., Roos, I. and Crompton, M. (1987) Biochemistry **26**, 246–254
10 McCormack, J. G., Halestrap, A. P. and Denton, R. M. (1990) Physiol. Rev. **70**, 391–425
11 Denton, R. M. and McCormack, J. G. (1985) Am. J. Physiol. **249**, E543–E554
12 Hansford, R. G. (1991) J. Bioenerg. Biomembr. **23**, 823–854
13 Crompton, M. (1985) Curr. Top. Membr. Transp. **25**, 231–276
14 Miyata, H., Silverman, H. S., Sollott, S. J., Lakatta, E. G. and Stern, H. D. (1991) Am. J. Physiol. **261**, H1123–H1134
15 Griffiths, E. J., Stern, M. D. and Silverman, H. S. (1997) Am. J. Physiol. **273**, C37–C44
16 Robb-Gaspers, L. D., Rutter, G. A., Burnett, P., Hajnorsky, G., Denton, R. M. and Thomas, A. P. (1998) Biochim. Biophys. Acta **1366**, 17–32
17 Duchen, M. R. (1999) J. Physiol. (London) **516**, 1–17
18 Richter, C. (1993) FEBS Lett. **325**, 104–107
19 Richter, C., Schweizer, M., Cossarizza, A. and Franchesi, C. (1996) FEBS Lett. **378**, 107–110
20 Nishiki, K., Erecinska, M. and Wilson, D. F. (1978) Am. J. Physiol. **234**, C73–C81
21 Williams, J. N. (1968) Biochim. Biophys. Acta **162**, 175–181
22 Erecinska, M. and Wilson, M. (1982) J. Membr. Biol. **70**, 1–16
23 Nicholls, D. G. (1978) Biochem. J. **176**, 463–474
24 Miyata, H., Lakatta, E. G., Stern, M. D. and Silverman, H. S. (1992) Circ. Res. **71**, 605–613
25 Nicholls, D. G. and Brand, M. D. (1980) Biochem. J. **188**, 113–118
26 LeFurgey, A., Ingram, P. and Lieberman, M. (1988) Cell Calcium **9**, 219–235
27 Murphy, E., Jacob, R. and Lieberman, M. (1985) J. Mol. Cell. Cardiol. **17**, 221–231
28 Broderick, R. and Somlyo, A. P. (1987) Circ. Res. **61**, 525–530
29 Hunter, P. R. and Haworth, R. A. (1979) Arch. Biochem. Biophys. **195**, 468–477
30 Crompton, M. and Costi, A. (1988) Eur. J. Biochem. **178**, 489–501
31 Crompton, M. and Costi, A. (1990) Biochem. J. **266**, 33–39
32 Ginsburg, H. and Stein, W. D. (1987) J. Membr. Biol. **96**, 1–10
33 Al Nasser, I. and Crompton, M. (1986) Biochem. J. **239**, 19–29
34 Gunter, T. E. and Pfeiffer, D. R. (1990) Am. J. Physiol. **258**, C755–C786
35 Ichas, F., Jouaville, L. S. and Mazat, J. P. (1997) Cell **89**, 1145–1153
36 Al Nasser, I. and Crompton, M. (1986) Biochem. J. **239**, 31–40
37 Huser, J., Rechenmacher, C. E. and Blatter, L. A. (1998) Biophys. J. **74**, 2129–2137
38 Bernardi, P., Broekemeier, K. M. and Pfeiffer, D. R. (1994) J. Bioenerg. Biomembr. **26**, 509–517
39 Le Quoc, K. and Le Quoc, D. (1988) Arch. Biochem. Biophys. **265**, 249–257
40 Halestrap, A. P., Kerr, P. M., Javadov, S. and Woodfield, K. Y. (1998) Biochim. Biophys. Acta **1366**, 79–94
41 Brustovetsky, N. and Klingenberg, M. (1996) Biochemistry **35**, 8483–8488
42 Ruck, A., Dolder, M., Wallimann, T. and Brdiczka, D. (1998) FEBS Lett. **426**, 97–101
43 Duchen, M., McGuinness, O. M., Brown, L. and Crompton, M. (1993) Cardiovasc. Res. **27**, 1790–1794
44 Halestrap, A. P., Woodfield, K. Y. and Connern, C. P. (1997) J. Biol. Chem. **272**, 3346–3354
45 Majima, E., Yamaguchi, N., Chuman, H., Shinohara, Y., Ishida, M., Goto, S. and Terada, H. (1998) Biochemistry **37**, 424–432
46 Crompton, M., McGuinness, O. M. and Nazareth, W. (1992) Biochim. Biophys. Acta **1101**, 214–217
47 McGuinness, O. M., Yafei, N., Costi, A. and Crompton, M. (1990) Eur. J. Biochem. **194**, 671–679
48 Doerner, A., Pauschinger, M., Badorff, A., Noutsias, M., Giessen, S., Schulze, K., Bilger, J., Rauch, U. and Schultheiss, H. P. (1997) FEBS Lett. **414**, 258–262
49 Crompton, M., Ellinger, H. and Costi, A. (1988) Biochem. J. **255**, 357–360
50 Halestrap, A. P. and Davidson, A. M. (1990) Biochem. J. **268**, 153–160
51 Griffiths, E. J. and Halestrap, A. P. (1991) Biochem. J. **274**, 611–614
52 Nicolli, A., Basso, E., Petronilli, V., Wenger, R. M. and Bernardi, P. (1996) J. Biol. Chem. **271**, 2185–2192
53 Andreeva, L., Tanveer, A. and Crompton, M. (1995) Eur. J. Biochem. **230**, 1125–1132
54 Tanveer, A., Virji, S., Andreeva, A., Totty, N., Hsuan, J. J., Ward, J. M. and Crompton, M. (1996) Eur. J. Biochem. **238**, 166–172
55 Crompton, M. and Andreeva, L. (1994) Biochem. J. **302**, 181–185
56 Andreeva, L. and Crompton, M. (1994) Eur. J. Biochem. **221**, 261–268
57 Szabo, I and Zoratti, M. (1991) J. Biol. Chem. **266**, 3376–3379
58 Crompton, M., Virji, S. and Ward, J. M. (1998) Eur. J. Biochem. **258**, 729–735
59 Woodfield, K, Ruck, A., Brdiczka, D. and Halestrap, A. P. (1998) Biochem. J. **336**, 287–290
60 Rojo, M. and Wallimann, T (1994) Biochim. Biophys. Acta **1187**, 360–367
61 Block, M. R. and Vignais, P. V. (1986) Biochemistry **25**, 374–379
62 Connern, C. P. and Halestrap, A.P (1992) Biochem. J. **284**, 381–385
63 Johnson, N., Virji, S., Ward, J. M. and Crompton, M. (1999) Eur. J. Biochem., in the press
64 Moynagh, P. N. (1995) Essays Biochemistry **30**, 1–14
65 McCabe, E. R. B. (1994) J. Bioenerg. Biomembr. **26**, 317–321
66 Beutner, G., Ruck, A., Riede, B., Welte, W. and Brdiczka, D. (1996) FEBS Lett. **396**, 189–195
67 Beutner, G., Ruck, A., Riede, B. and Brdiczka, D. (1998) Biochim. Biophys. Acta **1368**, 7–18
68 Marzo, I., Brenner, C., Zamzami, N., Susin, S. A., Beutner, G., Brdiczka, D., Remy, R., Xie, Z., Reed, J. C. and Kroemer, G. (1998) J. Exp. Med. **187**, 1261–1271
69 Lin, L., Hasumi, H. and Brandts, J. F. (1988) Biochim. Biophys. Acta **956**, 256–266

70 Matouschek, A., Rospert, S., Schmid, K., Glick, B. S. and Schatz, G. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 6319–6323
71 Lodish, H. F. and Kong, N. (1991) J. Biol. Chem. **266**, 14835–14838
72 Jaschke, A., Mi, H. and Tropschug, M. (1998) J. Mol. Biol. **277**, 763–769
73 Ratajczk, T., Carello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L. and House, A. K. (1993) J. Biol. Chem. **263**, 13187–13192
74 Wu, J., Matunis, M. J., Kraemer, D., Blobel, G. and Coutavas, E. (1995) J. Biol. Chem. **270**, 14209–14219
75 Holloway, M. P. and Bram, R. J. (1998) J. Biol. Chem. **273**, 16346–16350
76 Mikol, V., Kallen, J. and Walkinshaw, M. D. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 5183–5186
77 Alberg, D. G. and Schreiber, S. L. (1993) Science **262**, 248–250
78 Kakilis, L. T. and Armitage, I. M. (1994) Biochemistry **33**, 1495–1501
79 Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., Klee, C. B. and Schreiber, S. L. (1992) Biochemistry **31**, 3896–3901
80 Crompton, M., Virji, S., Doyte, V., Johnson, N. and Ward, J. M. (1999) Biochem. Soc. Symp., in the press
81 Theriault, Y., Logan, T. M., Meadows, R., Yu, L., Olejniczak, E. T., Holzman, T. E., Simmer, R. L. and Fesik, S. W. (1993) Nature (London) **361**, 88–91
82 Columbini, M., Yeung, C. L., Tung, J. and Konig, T. (1987) Biochim. Biophys. Acta **905**, 279–286
83 Rostovtsera, T. and Columbini, M. (1997) Biophys. J. **72**, 1954–1962
84 Zoratti, M. and Szabo, I. (1995) Biochim. Biophys. Acta **1241**, 139–176
85 Petronilli, V., Szabo, I. and Zoratti, M. (1989) FEBS Lett. **259**, 137–143
86 Szabo, I., Bernardi, P. and Zoratti, M. (1992) J. Biol. Chem. **267**, 2940–2946
87 Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabo, I. and Zoratti, M. (1992) J. Biol. Chem. **267**, 2934–2939
88 Lohret, T. A., Murphy, R. C., Drgon, T. and Kinnally, K. W. (1996) J. Biol. Chem. **271**, 4846–4849
89 Crompton, M., Costi, A. and Hayat, L. (1987) Biochem. J. **245**, 915–918
90 Nazareth, W., Yafei, N. and Crompton, M. (1991) J. Mol. Cell. Cardiol. **23**, 1351–1354
91 Crompton, M. and Andreeva, L. (1993) Basic Res. Cardiol. **88**, 513–523
92 Broekemeier, K. M., Carpenter-deyo, L., Reed, J. C. and Pfeiffer, D. R. (1992) FEBS Lett. **304**, 192–194
93 Imberti, R., Nieminen, A.-L, Herman, B. and Lemasters, J.J (1992) Res. Commun. Chem. Pathol. Pharmacol. **78**, 27–38
94 Kass, G. E. N., Juedes, M. J. and Orrenius, S. (1992) Biochem. Pharm. **44**, 1995–2003
95 Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. L. and Farber, J. L. (1993) J. Biol. Chem. **268**, 13791–13798
96 Shimizu, S., Kamiike, W., Hatanaka, N. and Miyata, T. (1994) Transplantation **57**, 1526–1536
97 Fujii, Y., Johnson, M. E. and Gores, G. J. (1994) Hepatology **20**, 177–185
98 Griffiths, E. J. and Halestrap, A. P. (1993) J. Mol. Cell. Cardiol. **25**, 1461–1469
99 Kristian, T. and Siesjo, B. K. (1998) Stroke **29**, 705–718
100 Niemenen, A. L., Petrie, T. G., Lemasters, J. J. and Selman, W. R. (1996) Neuroscience **75**, 993–997
101 Price, E. R., Jin, M., Lim, D., Pati, S., Walsh, C. T. and McKean, F. D. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3931–3935
102 Schneider, H., Charara, N., Schmitz, R., Wehrli, S., Mikol, V., Zurini, M. G. M., Quesniaux, V. J. F. and Movva, N. R. (1994) Biochemistry **33**, 8218–8224
103 Mi, H., Kops, O., Zimmerman, E., Jaschke, A. and Tropschug, M. (1996) FEBS Lett. **398**, 201–205
104 Kieffer, L. J., Seng, T. W., Li, W., Osterman, D. G., Handschumacher, R. E. and Bayney, R. M. (1993) J. Biol. Chem. **268**, 12303–12310
105 Wang, B. B., Hayenga, K. J., Payan, D. G. and Fisher, J. M. (1996) Biochem. J. **314**, 313–319
106 Rinfret, A., Collins, C., Menad, R. and Andersen, S. K. (1994) Biochemistry **33**, 1668–1673
107 Wu, J., Matunis, M. J., Kraemer, D., Blobel, G. and Coutavas, E. (1995) J. Biol. Chem. **270**, 14209–14213
108 Griffiths, E. J. and Halestrap, A. P. (1995) Biochem. J. **307**, 93–98
109 Kerr, P. M., Suleiman, M.-S. and Halestrap, A. P. (1999) Am. J. Physiol. **276**, H496–H502
110 Niemenen, A.-L., Saylor, A. K., Tesfai, S. A., Herman, B. and Lemasters, J. J. (1995) Biochem. J. **307**, 99–106
111 Zahrebelski, G., Niemenen, A.-L., Al-Ghoul, K., Qian, T., Herman, B. and Lemasters, J. J. (1995) Hepatology **21**, 1361–1372
112 Qian, T., Niemenen, A.-L., Herman, B. and Lemasters, J. J. (1997) Am. J. Physiol. **273**, C1783–C1792
113 Petronilli, V., Miotto, G., Colonna, R. and Bernardi, P. (1997) Biophys. J. **72**, 210a (abstr.)
114 Niemenen, A.-L., Byrne, A. M., Herman, B. and Lemasters, J. J. (1997) Am. J. Physiol. **272**, C1286–C1294
115 Steenbergen, C., Murphy, E., Watts, J. A. and London, R. E. (1990) Circ. Res. **66**, 135–146
116 Jennings, R. B. and Steenbergen, C. (1985) Annu. Rev. Physiol. **47**, 727–749
117 Vincent, M.-F, van den Bergh, G. and Hers, H. (1982) Biochem. J. **202**, 117–123
118 Kammermeier, H., Schmidt, P. and Jungling, E. (1982) J. Mol. Cell. Cardiol. **14**, 267–277
119 Novgorodov, S. E., Gudz, T. I., Milgrom, Y. M. and Brierley, G. P. (1992) J. Biol. Chem. **267**, 16274–16282
120 Klingenberg, M. (1976) in The Enzymes of Biological Membranes, vol. 3 (Martonosi, A., ed.), pp. 383–438, Plenum Press, New York
121 Chien, K. R. and Engler, R. (1990) in Calcium and the Heart (Langer, G. A., ed.), pp. 333–354, Raven Press, New York
122 Piper, H. M., Siegmund, B., Yu, V. L. and Schluter, K.-D. (1993) Basic Res. Cardiol. **88**, 471–482
123 Scholz, W. and Albus, V. (1993) Basic Res. Cardiol. **88**, 443–455
124 Murphy, E., Perlman, M., London, R. E. and Steenbergen, C. (1991) Circ. Res. **68**, 1250–1258
125 Allshire, A., Piper, M. H., Cuthbertson, K. S. R. and Cobbold, P. H. (1987) Biochem. J. **244**, 381–385
126 Miyamae, M., Camucho, S. A., Weiner, M. W. and Schemeueredo, F. M. (1996) Am. J. Physiol. **271**, H2145–H2153
127 McCord, J. M. (1985) N. Engl. J. Med. **312**, 159–163
128 Marklund, S. L. (1988) J. Mol. Cell. Cardiol. **20** (Suppl. II), 23–30
129 Gutteridge, J. M. and Halliwell, B. (1993) Arch. Biochem. Biophys. **283**, 223–226
130 Beetsch, J. W., Parks, T. S., Dugan, L.L, Shah, A. R. and Gidday, J. M. (1998) Brain Res. **786**, 89–95
131 Zingarelli, B., Salzman, A. L. and Szabo, C. (1998) Circ. Res. **83**, 85–94
132 Massberg, S., Enckers, G., Leiderer, R., Eisenmenger, S., Vestweber, D., Krambach, F. and Messmer, K. (1998) Blood **92**, 507–515
133 Reoh, G., Pratico, D., Juliano, L., Pulcinelli, F. M., Ghiselli, A., Pignatelli, P., Colavita, A.R and Fitzgerald, G. A. (1997) Circulation **95**, 885–891
134 Richter, C. (1988) FEBS Lett. **241**, 1–5
135 Lenaz, G. (1998) Biochim. Biophys. Acta **1366**, 53–67
136 Kowaltowski, A. J., Castilho, R. F. and Vercesi, A. E. (1996) FEBS Lett. **378**, 150–152
137 Nicotera, P., Bellomo, G. and Orrenius, S. (1992) Annu. Rev. Pharm. Toxicol. **32**, 449–470
138 Turrens, J. F., Freeman, B. A., Levit, J. G. and Crapo, J. D. (1982) Arch. Biochem. Biophys. **217**, 401–410
139 Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S. and Bernardi, P. (1994) J. Biol. Chem. **269**, 16638–16642
140 Chernyak, B. V. and Bernardi, P. (1996) Eur. J. Biochem. **238**, 623–630
141 Haworth, R. A. and Hunter, D. R. (1980) J. Membr. Biol. **54**, 231–236
142 Leyssens, A., Nowicky, A. V., Patterson, L., Crompton, M. and Duchen, M. R. (1996) J. Physiol. (London) **496.1** 111–128
143 Petronilli, V., Cola, C. and Bernardi, P. (1993) J. Biol. Chem. **268**, 1011–1016
144 Petronilli, V., Cola, C., Massari, S., Colonna, R. and Bernardi, P. (1993) J. Biol. Chem. **268**, 21939–21945
145 Bernardi, P., Veronese, P. and Petronilli, V. (1993) J. Biol. Chem. **268**, 1005–1011
146 Kowaltowski, A. J., Netto, L. E. and Vercesi, A. E. (1998) J. Biol. Chem. **273**, 12766–12771
147 Scorrano, L., Petronilli, V. and Bernardi, P. (1997) J. Biol. Chem. **272**, 12295–12302
148 Nicolli, A., Petronilli, V. and Bernardi, P. (1993) Biochemistry **32**, 4461–4465
149 Kumar, S. and Colus, P. A. (1999) Trends Biochem. Sci. **24**, 1–4
150 Zou, H., Henzel, W. J., Liu, X., Lutschg, A. and Wang, X. (1997) Cell **90**, 405–413
151 Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell **86**, 147–157
152 Srinivasula, S., Ahmad, M., Fernandez-Alnemri, T. and Alnemri, E. (1998) Mol. Cell **1**, 949–957
153 Pan, G., O'Rourke, K. and Dixit, V. (1998) J. Biol. Chem. **273**, 5841–5845
154 Reed, J. C. (1997) Cell **91**, 559–562
155 Luce, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Cell **94**, 481–490
156 Kannt, A., Lancaster, C. R. D. and Michel, H. (1998) J. Bioenerg. Biomembr. **30**, 1–6
157 Li, H., Zhu, H., Xu, C. and Yuan, J. (1998) Cell **94**, 491–501
158 Yoshida, H., Kong, Y.-Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M. and Mak, T. (1998) Cell **94**, 739–750
159 Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K.-M., Krammer, P. H. and Peter, M. E. (1998) EMBO J. **17**, 1675–1687
160 Mancini, M., Nicholson, D. W., Roy, S., Thornberry, N. A., Peterson, E. P., Casciola-Rosen, L. A. and Rosen, A. (1998) J. Cell Biol. **140**, 1485–1495

161 Susin, S., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M. C., Alzari, P. and Kroemer, G. (1999) J. Exp. Med. **189**, 381–394
162 Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. and Kroemer, G. (1996) J. Exp. Med. **184**, 1331–1342
163 Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, F., Geuskens, M. and Kroemer, G. (1996) J. Exp. Med. **184**, 1155–1166
164 Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M. et al. (1999) Nature (London) **397**, 441–450
165 Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J. and Shore, G. C. (1993) J. Biol. Chem. **268**, 25265–25268
166 Hsu, Y.-T, Wolter, K. G. and Youle, R. J. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 3668–3672
167 Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J. and Shore, G. C. (1998) J. Cell Biol. **143**, 207–215
168 Rosse, T., Olivier, R., Monney, L., Ragen, M., Conus, S., Fellay, I., Jansen, B. and Borner, C. (1998) Nature (London) **391**, 496–500
169 Mahajan, N. P., Linder, K., Berry, G., Gordon, G. W., Heim, R. and Herman, B. (1998) Nat. Biotechnol. **16**, 547–553
170 Munchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thopson, C. B., Wong, S.-L., Ng, S.-C. and Fesik, S. W. (1996) Nature (London) **381**, 335–341
171 Antonsson, B., Conti, F., Gavatta, A. M., Montessait, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J., Mazzei, G. et al. (1997) Science **277**, 370–379
172 Jurgensmeier, J. M., Xie, S., Deveraux, Q., Ellerby, L., Bredesen, D. and Reed, J. C. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 4997–5002
173 Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacher, P. T. and Thompson, C. B. (1997) Cell **91**, 627–637
174 Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M. J. and Kroemer, G. (1995) Exp. Med. **183**, 1533–1542
175 Kantrow, S. R. and Piantadosi, C. S. (1997) Biochem. Biophys. Res. Commun. **232**, 669–671
176 Ellerby, H. M., Martin, S. J., Ellerby, L. M., Naiem, S. S., Rabizadeh, S., Salvesen, G. S., Casiano, C. A., Cashman, N. R., Green, D. R. and Bredesen, D. E. (1997) J. Neurosci. **17**, 6165–6178
177 Friberg, H., Ferrand-Drake, M., Bengtsson, F., Halestrap, A. P. and Wieloch, T. (1998) J. Neurosci. **18**, 5151–5159
178 Marzo, I., Brenner, C., Zamzami, N., Jurgenmeier, J. M., Susin, S. A., Vieira, H. L. A., Prevost, M.-C., Xie, Z., Matsuyama, S., Reed, J. C. and Kroemer, G. (1998) Science **281**, 2027–2035
179 Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H. and Tsujimoto, Y. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 14681–14686
180 De Jong, D., Prins, F. A., Mason, D. Y., Reed, J. C., van Ommen, G. B. and Kluin, P. M. (1994) Cancer Res. **54**, 256–260
181 Lemasters, J. J., Niemenen, A. L., Qian, T., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A. and Herman, B. (1998) Biochim. Biophys. Acta **1366**, 177–196
182 Bossy-Wetzel, E., Newmeyer, D. D. and Green, D. R. (1998) EMBO J. **17**, 37–49
183 Charriant-Marlangue, C., Margaill, T., Borrega, F., Plotkine, M. and Ben-Ari, Y. (1996) Eur. J. Pharmacol. **310**, 137–140
184 Beilharz, E. J., Williams, C. E., Dragunow, M., Sirimanne, E. S. and Gluckmann, P. D. (1995) Mol. Brain Res. **29**, 1–14
185 Veinot, J. P., Gattinger, D. A. and Fliss, H. (1997) Hum. Pathol. **28**, 485–492
186 Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A. and Nicotera, P. (1995) Neuron **15**, 961–973
187 Bonfoco, E., Kraine, D., Ankarcrona, M., Nicotera, P. and Lipton, S. A. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7162–7166
188 Leist, M. and Nicotera, P. (1997) Biochem. Biophys. Res. Commun. **239**, 1–9
189 Kass, G. E. N., Eriksson, J. E., Weis, M., Orrenius, S. and Chow, S. C. (1996) Biochem. J. **318**, 749–752
190 Leist, M., Single, B., Castaldi, A. F., Kubule, S. and Nicotera, P. (1997) J. Exp. Med. **185**, 1481–1486
191 Tuena de Gomez-Puyou, M., Sandoval, F., Garcia, J. J. and Gomez-Puyou, A. (1998) Eur. J. Biochem. **255**, 303–308
192 Rutter, G. A., Burnett, P., Rizzuto, R., Brini, M., Murgia, M., Pozzan, T., Tavare, J. M. and Denton, R. M. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 5489–5494
193 Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Science **262**, 744–747
194 Simpson, P. B. and Russel, J. T. (1996) J. Biol. Chem. **271**, 33493–33501
195 Rizzuto, R., Pinton, P., Carrington, W., Fay, F., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. and Pozzan, T. (1998) Science **280**, 1763–1766
196 Bootman, M., Niggli, E., Berridge, M. and Lipp, P. (1997) J. Physiol. (London) **499**, 300–314
197 Duchen, M. R., Leyssens, A. and Crompton, M. (1998) J. Cell Biol. **142**, 975–988
198 Jacobson, D. and Duchen, M. R. (1998) J. Physiol. (London) **506**, 75P
199 Jouaville, L. S., Ichas, F., Holmuhamedov, E. L., Comacho, P. and Lechleiter, J. D. (1995) Nature (London) **377**, 341–348
200 Perez-Pinzon, M. A., Xu, G. P., Born, J., Lorenzo, J., Busto, R., Rosenthal, M. and Sick, T. L. (1999) J. Cereb. Blood Flow Metab. **19**, 39–43
201 Papadopuotos, V. (1993) Endocrin. Rev. **14**, 222–240
202 McEnery, M. W., Snowman, A. M., Trifiletti, R. R. and Snyder, S. H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3170–3174
203 Vance, J. E. (1990) J. Biol. Chem. **265**, 7248–7256
204 Ardail, D., Lerme, F. and Louisot, P. (1991) J. Biol. Chem. **266**, 7978–7981
205 Skulachev, V. P. (1990) J. Membr. Biol. **114**, 97–112
206 Konstantinova, S. A., Mannella, C. A., Skulachev, V. P. and Zorov, D. (1995) J. Bioenerg. Biomembr. **27**, 93–100
207 Fleckenstein, A., Janke, J., Doring, H. J. and Leder, O. (1974) Recent Adv. Stud. Card. Struct. Metab. **4**, 563–568
208 Choi, D. W. and Rothman, S. M. (1990) Annu. Rev. Neurosci. **13**, 171–182